

IN VITRO ANTICANCER, ANTIMICROBIAL, AND ANTIOXIDANT POTENTIALS OF *CHAMAECRISTA ROTUNDIFOLIA* VAR. *GRANDIFLORA* (AUTHORITY) LEAF EXTRACTS FROM NIGERIA

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ABSTRACT

The anticancer, antimicrobial, and antioxidant activities of *Chamaecrista rotundifolia* var. *grandiflora* (Ch.R) were evaluated for an insight into its potential as an alternative and complementary treatment. Dried leaves of Ch.R were extracted in 70% methanol (MECh.R) and partitioned into n-hexane (HFCh.R), dichloromethane (HFCh.R), chloroform (CFCh.R), and ethyl acetate (EAFCh.R). The anticancer activity of the crude extract and its fractions on cancer cell lines of HeLa, U-87, MCF-7, and healthy cells (HEK-293) was determined using MTT colorimetric assay. The micro-broth dilution method was used to analyse the antibacterial activity of Gram-positive and Gram-negative bacteria, while DPPH and CUPRAC methods were used to evaluate the antioxidant activity. The Folin-Ciocalteu and the aluminum chloride methods were used for the determination of total phenolic (TPC) and total flavonoid contents (TFC), respectively. All fractions of Ch.R inhibited the cell growth of all cancer cell lines. DFCh.R showed the highest inhibitory effect on the HeLa cell line ($IC_{50} = 7.94 \pm 1.34 \mu\text{g/mL}$) as well as on U-87 cells ($IC_{50} = 29.17 \pm 1.17 \mu\text{g/mL}$). HFCh.R also showed noticeable anti-proliferative potency on HeLa cells ($IC_{50} = 15.67 \pm 1.14 \mu\text{g/mL}$). HFCh.R exhibited the highest antimicrobial activity against *Chromobacterium violaceum* 12472 with an MIC value of 250 $\mu\text{g/mL}$. MECh.R has the highest antioxidant activity with a cupric reducing capacity of 420 mg TE/g DW and an IC_{25} of 443.7 $\mu\text{g/mL}$. Correlation between TPC, TFC, and antioxidant activity reveals a significant association and that phenolics and flavonoids could be responsible for the antioxidant activity observed in this plant. Overall, our results reveal the anticancer and anti-oxidative potential of Ch.R leaf and their promising fractions.

INTRODUCTION

Plants form a major part of our natural ecosystem and are an important resource, especially in sub-Saharan countries, to combat serious diseases. About 85% of the world's population depends on plants as a source of medicine [1]. In anticancer therapy, 60% of active anti-tumour compounds are derived from medicinal plants and other natural resources [2]. Resistance to drugs is still a major obstacle in cancer therapy. Tests for the cytotoxic and antioxidant properties of plants are a research area that is building much attention for the possible discovery of new substances for the prevention or treatment of cancer through phytochemicals and phytotherapy. Natural antioxidants of plant origin like vitamin C, polyphenols, and carotenoids have a wide range of pharmacological activities including free radical scavenging and preventing DNA damage and aging-related diseases [3].

Chamaecrista rotundifolia is a leguminous shrub or subshrub perennial plant that grows up to about 1 m. It is used as a ley in Nigeria and grown as a forage in China [4]. Though quite underexploited for its biological activity, its anti-infective activities (antibacterial, antiviral and antiparasitic) were

recently reported leading to the isolation of a few chromone derivatives [5-7]. *Chamaecrista rotundifolia* var. *grandiflora* (Ch.R) is a variety with distinct morphological features including larger flowers, the largest petal (up to 17 mm long), up to 7 fertile stamens, the longest fertile anther (~11.5 mm), and style length up to 8.5 mm. Though Ch.R has been used in malaria treatment in the Benin Republic, no scientific elucidation of its biological activity has been reported. Therefore, this study aims to investigate the potential anticancer, antibacterial, and antioxidant properties of *Chamaecrista rotundifolia* var. *grandiflora*.

RESULTS AND DISCUSSION

Anticancer Activity

Chamaecrista species are known for their medicinal value and are used in folklore. Some taxa of *Chamaecrista* have been used to treat malaria, piles, syphilis, snake bites, and scorpion stings in Africa [8, 9]. A plant like *Chamaecrista absus* has been validated to possess antibacterial, antifungal, anti-inflammatory, and antidiabetic potentials [10]. However, Ch.R taxon is yet to be studied for its ethnomedicinal applications.

Crude methanol extract and its fractions showed an inhibitory effect on HeLa, U87-MG, and MCF-7 cell lines (Table 1). Anticancer activities of the extract/fractions were concentration-dependent. However, only Hexane (HFCh.R) and Dichloromethane (DFCh.R) fractions possessed IC_{50} values below 30 $\mu\text{g/mL}$. The most active fraction on HeLa was DFCh.R (IC_{50} 7.94 $\pm$ 1.34 $\mu\text{g/mL}$) followed by HFCh.R (IC_{50} 15.67 $\pm$ 1.14 $\mu\text{g/mL}$). None had an IC_{50} of < 30 $\mu\text{g/mL}$ on the MCF-7 cell line (Table 1 and Fig. 1). Only the DFCh.R fraction had an IC_{50} less than 30 $\mu\text{g/mL}$ on U87-MG cells (IC_{50} = 29.17 $\pm$ 1.17 $\mu\text{g/mL}$). These findings show that the extracts and fractions of Ch.R have anticancer potential. Further, the extracts/fractions show concentration-dependent growth inhibition. The hexane fraction of Ch.R shows significant antiproliferative activity at 50 $\mu\text{g/mL}$ on cancerous cells studied (HeLa: 80%, MCF-7: 62.5% and U-87: 79%) while it has moderate activity on HEK-293 (58.8%). In the present study, Ch.R taxon had better antiproliferative

action on cancerous cells when compared to the taxa of *Chamaecrista* as reported by [11]. According to the American National Cancer Institute (NCI), the criteria of cytotoxicity (anticancer) for crude extracts is an IC_{50} < 30 $\mu\text{g/mL}$ after an exposure time of 72 h in a preliminary assay [12]. Going by this, it can be said that hexane and DCM fractions of Ch.R have significant anticancer activity on HeLa cells since their IC_{50} values fell below 30 $\mu\text{g/mL}$. Furthermore, according to the selectivity index (SI) value, the dichloromethane fraction for HeLa cells has the greatest selectivity index value among all the extracts/fractions. In this study, the selectivity index (SI) was used to evaluate the cytotoxicity of the extract/fractions against cancerous (HeLa, U87-MG, MCF-7) and normal kidney cells (HEK293) (Table 2). High SI values indicated that cancer cells would be killed at a higher rate than noncancerous cells. While the DFCh.R fraction for HeLa cells had the greatest selectivity index value among all samples tested, doxorubicin showed narrow selectivity only towards U87-MG cells.

Table 1: IC_{50} values ($\mu\text{g/mL}$) of the extract and fractions of *Chamaecrista rotundifolia* var. *grandiflora* on various cell lines.

IC_{50} value ($\mu\text{g/mL}$)				
Extracts/fractions	HeLa	U87-MG	MCF - 7	HEK - 293
MECh.R	187.50 $\pm$ 1.11	261.22 $\pm$ 1.05	288.40 $\pm$ 1.09	131.22 $\pm$ 1.09
HFCh.R	15.67$\pm$1.14	41.02 $\pm$ 1.29	42.17 $\pm$ 1.11	11.48 $\pm$ 1.22
DFCh.R	7.94$\pm$1.34	29.17 $\pm$ 1.17	59.70 $\pm$ 1.12	11.22 $\pm$ 1.07
CFCh.R	102.33 $\pm$ 1.08	149.62 $\pm$ 1.10	269.15 $\pm$ 1.75	47.32 $\pm$ 1.10
EAFCh.R	79.43 $\pm$ 1.05	91.20 $\pm$ 1.10	60.26 $\pm$ 1.06	38.19 $\pm$ 1.11
Doxorubicin	1.18 $\pm$ 0.64	0.55 $\pm$ 0.62	1.03 $\pm$ 0.66	0.90 $\pm$ 0.62

MECh.R = Methanol extract, HFCh.R = Hexane fraction, DFCh.R = Dichloromethane fraction, CFCh.R = Chloroform fraction, and EAFCh.R: Ethyl acetate fraction of *C. rotundifolia*,

Table 2: Selectivity index of the extract and fractions of *Chamaecrista rotundifolia* var. *grandiflora* on various cell lines.

Selectivity index*			
Extracts/fractions	HeLa	U87 - MG	MCF - 7
MECh.R	0.70	0.50	0.46
HFCh.R	0.73	0.28	0.27
DFCh.R	1.41	0.39	0.19
CFCh.R	0.46	0.32	0.18
EAFCh.R	0.48	0.42	0.63
Doxorubicin	0.76	1.64	0.87

*Selectivity index = IC_{50} for HEK-293 (healthy) cell / IC_{50} for each cancer cell

Antibacterial activity

Most of the bacterial strains, except *P. mirabilis*, were inhibited by at least one concentration of the fractions (Table 3). The lowest MIC value (250 $\mu\text{g/mL}$) was obtained with hexane fraction against *C. violaceum* (Table 4). Overall, the fractions were more effective against *C. violaceum* than other bacteria strains. MIC chosen is of concentrations that inhibited growth around 15% and above. The chloroform fraction had the highest growth inhibition rate (47.02%), followed by the dichloromethane fraction (43.94%). Surprisingly, the antibacterial effect of Ch.R taxon was more effective on Gram-negative bacteria than Gram-positive bacteria considering that Gram-negative bacteria are mostly resistant to antibiotics. This is in contrast with the extract of most medicinal plants. Rarely

do plants show antimicrobial activity against Gram-negative bacteria [13]. All fractions/extracts of Ch.R have a growth inhibition effect on the pathogen at certain concentrations. Hexane and dichloromethane fractions showed the most antibacterial activity. Chloroform fraction exhibited low MIC and percentage growth inhibition activity on bacteria. The fractions were most effective against *C. violaceum*, followed by *E. coli* and *P. aeruginosa*. However, the fractions show no antibacterial effect on *P. mirabilis*. Only hexane and dichloromethane fractions showed inhibitory effects on the growth of Gram-positive bacteria studied (*S. aureus* and *S. epidermidis*). Recently, Yang *et al.* [14, 15] reported the antibacterial activity of some chromones derivatives from the whole plant of *Chamaecrista rotundifolia*.

a) HFCh.R fraction

b) DFCh.R fraction

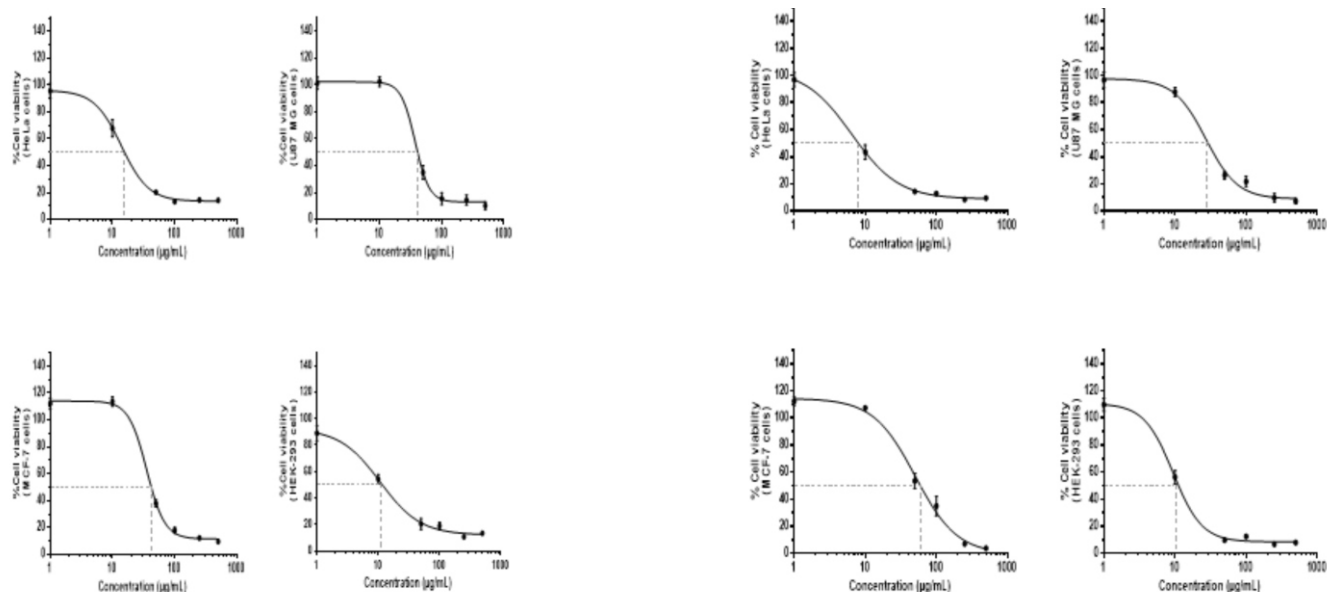


Figure 1. Chart showing inhibition of cancer cell proliferation by HFCh.R (a) and DFCh.R (b) fractions

HFCh.R = Hexane fraction, DFCh.R = Dichloromethane fraction of *Chamaecrista rotundifolia*. Information on absorbance and IC_{50} needs to be included.

Table 3: Percentage (%) growth inhibitory activity of fractions on pathogens

Plant	Fractions	Concentrations (µg/mL)	<i>S.</i> <i>aureus</i>	<i>S.</i> <i>epidermidis</i>	<i>P.</i> <i>aeruginosa</i>	<i>E.</i> <i>coli</i>	<i>P.</i> <i>mirabilis</i>	<i>C.</i> <i>violaceum</i>
Ch.R	HF	1000	26.91	14.75	18.61	NI	NI	27.09
		500	NI	NI	7.94	8.55	NI	33.59
		250	NI	NI	NI	NI	NI	38.51
		50	NI	NI	NI	NI	NI	5.29
	DF	1000	NI	17.65	24.24	30.61	NI	40.51
		500	NI	10.48	11.67	24.32	NI	43.94
		250	NI	NI	6.2	NI	NI	NI
		50	NI	NI	NI	16.27	NI	NI
	CF	1000	NI	NI	NI	13.24	NI	47.02
		500	NI	NI	NI	NI	NI	NI
		250	NI	NI	NI	NI	NI	NI
		50	NI	NI	NI	NI	NI	NI

NI: No inhibition, Ch. R: *Chamaecrista rotundifolia*, HF: Hexane fraction, DF: Dichloromethane fraction, CF: Chloroform fraction.

Table 4: Minimum inhibitory concentration (MIC) of fractions on treated pathogens

Microorganisms		Ch.R (µg/mL)			Amp	Gn	Kn
		HF	DF	CF			
<i>S. aureus</i>	29213	1000	ND	ND	ND	4	4
<i>S. epidermidis</i>	12228	ND	1000	ND	ND	2	2
<i>P. aeruginosa</i>	27853	1000	1000	ND	ND	256	ND
<i>E. coli</i>	25922	ND	500	ND	16	4	2
<i>P. mirabilis</i>	14153	ND	ND	ND	ND	16	8
<i>C. violaceum</i>	12472	250	500	1000	ND	32	4

ND: Not detected, Amp: Ampicillin, Gn: Geneticin, Kn: Kanamycin, Ch. R: *Chamaecrista rotundifolia*, HF: Hexane fraction, DF: Dichloromethane fraction, CF: Chloroform fraction.

Antioxidant Analysis

The ethyl acetate fraction of the methanolic extract (EAFCh.R) showed the highest TPC value (93.9 ± 0.06 mg GAE/g dry extract) compared to the ethyl acetate extract Ch.R (EAECh.R) 6.7 ± 0.625 mg GAE/g dry extract (Table 5). Also, the highest TFC value was observed in EAFCh.R (698 ± 5 mg QE/g dry extract) as the methanol extract (MECh.R) also had a noticeable TFC value (289 ± 5 mg QE/g dry extract) compared to the EAECh.R. The less TFC value (69 ± 5 mg QE/g dry extract) was observed in the non-polar fractions (HFCh.R and CFCh.). Although, there was no report on the TPC of Ch.R in the present study, a comparison with other members of the same genus showed that the plant had excellent TPC. The TPC of the methanolic extract of *Chamaecrista absus* var. *absus* was reported to be 7.56 ± 0.32 mg GAE/g dry extract by Sebei *et al* [16] and also that of *Chamaecrista hildebrandtii* to be 1.33 ± 0.07 mg/g TAE (tannic acid equivalent) [17]. Comparing these values with the TPC of the methanolic extract of Ch.R of the present study showed that Ch.R had much more phenolic content (34 ± 1.09 mg GAE/g dry extract). Likewise, the TFC value of extracts and fractions were reported in quercetin equivalent using the standard curve equation $y = 0.0002x + 0.0066$, $R^2 = 0.9924$. The total flavonoid content of Ch.R obtained from both the methanolic and ethyl acetate extracts was significantly higher than the total flavonoid content of *Chamaecrista hildebrandtii* (2.69 ± 0.33 mg CE/g DW) as previously reported by Odhiambo *et al.* [17] and *Chamaecrista absus* var. *absus* (1.113 ± 0.08 mg CE/g DW) [16]. This inter-species variation in the phenolics and flavonoids in *Chamaecrista* species is worthy of note. This variation could genetically influence the biosynthesis of phenolics in types and quantity [18]. The improved antioxidant capacity of the ethylacetate fraction from the methanol extract of Ch.R compared to the ethylacetate extract highlighted the phenolic extraction efficiency of Ch.R in methanol versus ethylacetate.

The highest cupric reducing capacity of extracts was observed in EAFCh.R at 1040 ± 10 mg TE/g DW. This was followed by

MECh.R extract which was at 420 ± 5 mg TE/g DW. The cupric reducing capacities of the fractions obtained from the methanolic extracts were in the order ethyl acetate > dichloromethane > hexane > chloroform (Table 5). The crude extract and fractions of Ch.R had significant cupric-reducing capacity. Results suggested that the cupric-reducing capacity of Ch.R is due to the ability of flavonoids to chelate Cu (II) as shown from the corresponding TFC and CUPRAC values. The cupric-reducing capacities of Ch.R had not been reported previously. The ethyl acetate fraction of the methanolic extract showed the best cupric reducing capacity, which was in line with TPC and TFC results. As recorded in TPC, TFC, and CUPRAC analyses, the ethyl acetate fraction of the methanolic extract (EAFCh.R) showed the highest DPPH radical scavenging activity (Figure 5). Overall, EAFCh.R had the best antioxidant ability. The hexane and chloroform fractions of Ch.R in the present study had poor antioxidant activity. The antioxidant capacity of Ch.R with DPPH was significantly lower than that of *Chamaecrista hildebrandtii* (IC_{50} 8.7 mg/mL) [17], but significantly higher than that of *Chamaecrista absus* var. *absus* with IC_{50} value of $16.78 \mu\text{g/mL}$ [16].

Phenolics and flavonoids have been known to act as free radical scavengers and are good indicators of the antioxidant activity of plant extracts or fractions [19]. Different literature reports indicate a linear correlation of total phenolic and flavonoid content with antioxidant capacity [20]. The correlation between TPC, TFC, and antioxidant activity is shown in Table 6 below. A high correlation between total phenolic and antioxidant capacity (DPPH, 100%; CUPRAC, 95.24%) was observed at a 95% confidence level. A high correlation between total flavonoid and antioxidant capacity (CUPRAC, 100%; DPPH, 95.64%) was also observed at a 95% confidence interval. By comparing the correlation percentages from the R-value, it is possible to suggest that phenolics and flavonoids could be responsible for the antioxidant activity of the extracts of the plants. The total phenolic and flavonoid content in the present study shows a significant correlation with the antioxidant activity of Ch.R.

Table 5: Summary of the antioxidant activities of Ch.R (mean values, n=3)

Sample Code	TPC (mg GAE/g dry weight of the extract)	TFC (mg QE/ g dry weight of the extract)	DPPH· Scavenging IC ₂₅ (µg/mL)	CUPRAC (mg TE/ g dry weight of the extract)
MECh.R	34.0±1.09	289±5	443.7	420±5
EAECh.R	6.7±0.625	142±0	1000	378±5
HFCh.R	Negative	168±0	ND	210±0
DFCh.R	2.1±0.129	118±10	1000	225±0
CFCh.R	Negative	69±5	ND	130±15
EAFCh.R	93.9±0.06	698±10	301.83	1040±10
Ascorbic Acid	—	—	20.9	—

MECh.R: Methanol extract, EAECh.R: Ethyl acetate extract, HFCh.R: Hexane fraction, DFCh.R: Dichloromethane fraction, CFCh.R: Chloroform fraction, and EAFCh.R: Ethyl acetate fraction of *C. rotundifolia* var. *grandiflora*, ND: Not detected, -: not included

Table 6. Linear relationship between TPC, TFC, and antioxidant activity

	Equation	R ²	p
TPCDPPH	y = 0.5075x + 76.644	1	<0.05
TPCUPRAC	y = 8.305x + 231.93	0.9524	<0.05
TFCDDPPH	y = - 2.5663x + 1439.6	1	<0.05
TFCUPRAC	y = 1.394x + 55.716	0.9564	<0.05

CONCLUSION

Our findings demonstrate for the first time the anticancer, antioxidant, and antibacterial properties of Ch.R. The result supports the ethnopharmacological use of Ch.R. The dichloromethane fraction has the most anticancer properties while the hexane fraction exhibited the most antibacterial activity. The fractions of Ch.R are most effective on Gram-negative bacteria. Ethyl acetate fraction demonstrated the best antioxidant properties and the antioxidant properties are due to the high phenolic and flavonoid content of Ch.R. We conclude that *Chamaecrista rotundifolia* var. *grandiflora* possesses ethnopharmacological properties and has the potential to combat cancer cells and pathogens. Further *in vitro* and *in vivo* studies are needed to evaluate active principles and understand the mechanism of action and possible side effects.

MATERIALS AND METHODS

Plant Materials, Identification, and Extraction

Dried, powdered plant materials of *Chamaecrista rotundifolia* var. *grandiflora* were collected from the University of Ilorin environs, authenticated and deposited at the Forest Herbarium Ibadan (FHI), Forest Research Institute Nigeria, with the voucher number FHI112837. Exactly, 250 g of the Ch.R leaves were macerated in 1.55 L of methanol (70%) and allowed to stand in an airtight round bottom flask for three (3) days with daily stirring. The extract was filtered using a vacuum filter and concentrated using a rotary evaporator (100 rpm, 55 °C; 5 h). Concentrated extract was then frozen (-77 °C; 24 h) and lyophilized to dryness (-58 °C; 24 h). Exactly, 2 g of crude extract was dissolved in distilled water (100 mL) and partitioned with equal volumes of n-hexane (H), dichloromethane (D), chloroform (C), and ethyl acetate (E) for liquid-liquid partitioning. Organic solvents were evaporated and concentrated *in vacuo* to obtain H, D, C, and E fractions of the crude extract. Stock solutions of crude extract and its fractions in various concentrations in dimethyl sulfoxide

(DMSO) were prepared and stored at -20°C until further analysis. Crude extract of ethyl acetate (EAE) was also prepared, in which 50 g Ch.R was macerated in 500 mL of ethyl acetate for 3 days with intermittent shaking. The EAE was filtered and then concentrated to dryness using a rotary evaporator. A stock solution of 100 mg/mL in DMSO was stored at -20°C until use for antioxidant activity tests.

MTT Anticancer Assay

Four different cell lines were used: HeLa (cervical cancer), U-87 (brain cancer), MCF-7 (breast cancer), and HEK-293 (immortalized human embryonic kidney cell). HeLa cells were grown and sub-cultured in Minimum Essential Medium (MEM), U87-MG cells were grown and sub-cultured in Dulbecco's Modified Eagle Medium/High Glucose, MCF-7 and HEK-293 cells were grown and sub-cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/F-12), which are supplemented with 10% Fetal Bovine Serum (FBS), 1% antibiotic and antimycotic (penicillin, streptomycin, and amphotericin B) in an incubator with 37°C and 5% CO₂. Cell culture reagents were purchased from Gibco (Carlsbad, CA, USA). MTT assay was performed according to Mosmann [21] with slight modifications. Exactly, 200 µL of cell suspension (1x10⁵ cells/mL) were seeded in a 96-well plate and incubated at 37°C for 24 h with 5% CO₂. After 24 h, the cell culture medium was removed from each well without disturbing the cells and 200 µL of different concentrations of crude extract (1, 10, 100, 250, 500, 1000 µg/mL) and fractions (1, 10, 50, 100, 250, 500 µg/mL) were treated with cells in each well for 48 h. Treatments were removed and cells were incubated with 30 µL MTT at a final concentration of 5 mg/mL in phosphate-buffered saline (PBS) at 37°C with 5% CO₂ for 4 h. After incubation, 150 µL of DMSO was added to dissolve the formazan crystals. The absorbance of each sample was measured at 540 nm by a microplate reader. Cell viability was calculated according to the formula:

$$\% \text{ Cell viability} = \frac{\text{absorbance of experimental group}}{\text{absorbance of control group}} \times 100$$

All test samples were measured in triplicate and the extracts' half-maximal inhibitory concentrations (IC₅₀) were calculated. Cells without treatment were used as control and DMSO as blank. Doxorubicin was used as a positive standard.

Antibacterial Assay

Six pathogenic ATCC bacterial strains were used for antibacterial analysis. Two of the strains were Gram-positive (*Staphylococcus aureus* 29213 and *Staphylococcus epidermidis* 12228) and four were Gram-negative (*Pseudomonas aeruginosa* 27853, *Escherichia coli* 25922, *Proteus mirabilis* 14153 and *Chromobacterium violaceum* 12472). All strains were grown in a liquid broth (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 30 °C and 180 rpm, only *E. coli* was grown at 37°C. Antibacterial activities of Hexane, Dichloromethane, and Chloroform fractions were evaluated according to the broth dilution assay in 96-well plates as described by Zgoda & Porter [22] with slight modifications. Four serial dilutions were prepared from the stock solution (250 mg/mL in DMSO) of samples with 100 µL LB, giving a final concentration of 50, 250, 500, and 1000 µg/mL in wells. Bacterial suspensions were adjusted to OD₆₀₀ = 0.1 with LB broth using fresh overnight culture, giving a total volume of 200 µL in each well. Two-fold serial dilutions of ampicillin, kanamycin, and gentamicin were used as standard antibiotics giving a final concentration range of 0.25- 256 µg/mL in wells. Bacterial cultures without treatment were used as negative controls and 0.4% of DMSO was used as vehicle solvent control. The plates were covered, carefully mixed, and incubated at 30 °C for 24 h (except for *E. coli* which was incubated at 37 °C). The absorbance of each sample was measured using an Epoch microplate spectrophotometer (Biotek, USA) at 600 nm. The MIC was defined as the lowest concentration that visibly inhibited the growth of microorganisms. The percentage of bacterial growth inhibition was determined using the formula below:

Where A is the absorbance.

Antioxidant Activity Assays

Determination of Total Phenolic Content (TPC)

The Folin-Ciocalteu's phenol reagent was used in this assay [23]. In a 1.5 mL microtube, 100 µL of the crude extracts (methanol and ethylacetate) and four fractions of methanol extract in a final concentration of 1000 µg/mL in DMSO was added, followed by 50

µL of 2N Folin-Ciocalteu's reagent (Sigma-Aldrich), and 300 µL of Na₂CO₃ solution in water (200 g/L) was added. Finally, 550 µL of distilled water was added into each tube, vortexed, and centrifuged at 1250 g for 5 min in a centrifuge with coolant (Sigma 3-30K). Thereafter, 200 µL of supernatant from each tube was applied to each well of a 96-well plate in triplicate. DMSO and water were used as blanks. Absorbance readings were taken at 725 nm. Gallic acid (Merck) was used as a standard and results were expressed as milligram Gallic acid equivalent (GAE) per gram dry extract using the equation obtained from the linear regression calibration curve of Gallic acid and the formula below:

$$\% \text{Growth Inhibition} = [(A_{\text{negative control}} - A_{\text{sample}}) / A_{\text{negative control}}] \times 100.$$

$$\text{TPC} = c_{\text{GA}} \times V/m$$

where, TPC = total phenolic content as mg GAE/g dry extract, c_{GA} = concentration of gallic acid obtained from calibration curve in mg/mL, V = volume of extract in mL, m = mass of extract in gram.

Determination of Total Flavonoid Content (TFC)

The aluminum complexation assay was used for the total flavonoid content (TFC) assay as described by Suantawee *et al.* [24]. Briefly, 100 µL of the sample was added to 400 µL of distilled water, followed by 30 µL of 5% NaNO₂ (Merck), and allowed to stand for 5 min. Then 30 µL of 10% AlCl₃ (Riedel-de Haen AG) was added and incubated for 1 min. Afterwards, 200 µL of NaOH (Sigma-Aldrich) was added and the final volume was completed to 1 mL with distilled water. Exactly 200 µL of each sample mixture was applied to each well of a 96-well plate in triplicates and read at 510 nm with a spectrophotometer. DMSO was used as control and Quercetin (1-1000 µg/mL) was used as standard. Linear regression calibration curve was plotted and TFC results were expressed as mg Quercetin equivalent (QE) per gram dry extract weight of sample from the standard curve of Quercetin and using the following formula:

$$\text{TFC} = c_{\text{Q}} \times V/m$$

where, TFC = total flavonoid content as mg QE/g dry extract, c_Q = concentration of quercetin obtained from calibration curve in mg/mL, V = volume of extract in mL, m = dry weight of the extract in gram

CUPRAC Assay

This assay was carried out as described by Hamad *et al.* [25] in a 24-well plate. The concentration of the samples in DMSO was the same as in the DPPH

assay. Trolox was used as the standard antioxidant while DMSO and ethanol as blanks. Exactly, 250 μ L of 0.01 M copper (II) chloride (MERCK) in water was applied to each well followed by 250 μ L of 7.5×10^{-3} M neocuproine (Sigma-Aldrich) in ethanol and then 250 μ L of 1 M ammonium acetate (BioMatik) in water. Then, 25 μ L of the sample was added to each well, followed by 250 μ L of distilled water. Tests were conducted in triplicates and the whole process was carried out in a shaker. Plates were kept in the dark for an hour. The absorbances were measured at 450 nm by using a BioTek Eon microplate spectrophotometer. Results were expressed as milligram Trolox equivalent (TE) antioxidant capacity per gram dry weight of the extract using the equation obtained from the calibration curve of Trolox and the following formula:

$$\text{TEAC} = c_T \times V/m$$

Where, TEAC = Trolox equivalent antioxidant capacity as mg TE/g dry extract, c_T = concentration of Trolox obtained from calibration curve in mg/mL, V = volume of extract in mL, m = mass of dry extract in gram

DPPH Assay

The DPPH radical scavenging activities of the crude extracts and the fractions were determined using a 96-well plate and the method described by Hamad *et al.* [25]. Different concentrations of extracts and fractions (500, 250, 100, 50, 10, and 1 μ g/mL) in DMSO were prepared and kept in microtubes (1.5 mL). Exactly, 40 μ L of each concentration of crude extracts and fractions were applied to a 96-well plate in triplicates and 160 μ L of 0.2 mM DPPH (Sigma-Aldrich) in methanol was applied to every well on the plate. The plate was left to stand in a dark chamber for 10 min and absorbance was recorded using BioTek Eon microplate spectrophotometer at 520 nm [25]. Ascorbic acid (SigmaUltra) in water was used as a positive control, while water was used as blank for reference antioxidant, and DMSO as test materials. Results were expressed as % DPPH Radical Scavenging using the formula:

$$\% \text{ DPPH Radical Scavenging} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

Data Analysis

Data were expressed as mean $\pm$ standard error of the mean. For half-maximal inhibitory concentrations (IC_{50}), nonlinear regression analysis was used and IC_{50} values were calculated with a sigmoidal dose-response curve.

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