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In Vitro Anti-MRSA (Methicillin-Resistant Staphylococcus Aureus) Activities of the Partitions and Fractions of the Crude Aqueous Leaf Extract of Chromolaena Odorata (King and Robinson)

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Abstract: *The aim of this study was to determine the in vitro anti-MRSA activities of the partitions and fractions of the crude aqueous leaf extract of Chromolaena odorata against clinical isolates of MRSA. Partitioning of the extract with chloroform gave two partitions. The aqueous partition (AP) gave a higher yield of 59.74% than the chloroform partition (CP) with 2.69%. However, the CP showed a higher anti-MRSA activity. The mean values of zones of inhibition at concentrations of 3.13-25.0mg/ml for the AP and CP were 9.43±1.33mm and 16.61±0.93mm respectively. The minimum inhibitory concentration was 12.5mg/ml for AP and 3.13mg/ml for CP. Column chromatography of CP resulted to fractions with variety of colours. Thin layer chromatography of these fractions gave ten fractions with the highest retention factor (Rf) value of 0.93 for fraction F10 and the least Rf value of 0.43 for fraction F8. At a concentration of 2.0mg/ml, fractions F2 and F3 gave the highest zones of inhibition of 22.5±0.05mm and 22.5±2.50mm respectively while F5 had the least activity of 8.0±8.0mm. This study suggests that C. odorata is a potential and promising plant that should be exploited for the management of MRSA diseases.*

Keywords: *Chromolaena odorata, Methicillin-resistant Staphylococcus aureus (MRSA), retention factor (Rf), phytochemicals.*

I. Introduction

Chromolaena odorata (L.) King and Robinson commonly known as Eupatorium odoratum L. is a perennial shrub that is a native to South and Central America and later introduced into tropical regions of Asia, Africa and the Pacific (1). It grows in pastures, marginal lands, open areas, dry deciduous forests and interior shrub jungles where it is highly competitive and does not let other flora grow. It is a menace in plantations, agriculture and other ecosystems. It suppresses young plantations, agricultural crops and smothers vegetation as it possesses allelopathic potentialities and growth inhibitors (2, 3, 4).

It contains a diverse range of secondary chemicals including flavonoids, terpenoids, alkaloids, tannins, steroids and saponins which produce a definite physiological action on the human body (5, 6). These chemicals are mainly responsible for the activities of the plant which include: wound healing, antioxidant and antimicrobial activities. It has immense medicinal and nutritional potentials only waiting for mankind to continue to tap and benefit from (7). In traditional medicine, C. odorata is used as: anti-spasmodic, anti-inflammatory, astringent, diuretic, hepatotropic, antihypertensive, anti-trypanosomal, antiprotozoal, antifungal and antibacterial agents (8, 9). It has been reported that between the years 1983 and 1994, the systematic screening of antibacterial plant extracts represents a continuous effort to find new compounds with the potential to act against multi-resistant bacteria. According to World Health Organization (WHO), medicinal plants would be the best source to obtain a variety of drugs. Researchers are increasingly turning their attention to folk medicine (10).

It has been reported that the crude ethanolic and aqueous leaf extracts of C. odorata have anti-MRSA (methicillin-resistant Staphylococcus aureus) activities (6). The primary benefits of using plants derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment.

The treatment of infections caused by MRSA has become more problematic since the bacterium is increasingly resistant to as many as twenty different antimicrobial compounds. These include the biocides that represent most of the available drug classes (11). MRSA infections have now become a major public health concern and its prevalence is also increasing globally (12).

A formulation prepared from the aqueous extract of the leaves of C. odorata has been licensed for clinical use in Vietnam for treatment of leech bites, soft tissue burns and wounds, skin infections and dento-

alveolitis (13, 14, 15, 1). Also in the southern part of Nigeria, the leaves of the plant are used for wound dressing, treatment of skin infection and to stop bleeding (16).

This suggests a need to isolate and evaluate the active constituents of C. odorata which can be used for the development of novel chemotherapeutic agents for the effective treatment of MRSA infections.

This work was carried out to determine the anti-MRSA activities of the partitions and fractions of crude aqueous leaf extract of C. odorata.

II. Materials And Methods

2.1 Identification of MRSA and MSSA (methicillin-susceptible S. aureus) isolates.

Eleven (11) clinical isolates (7 MRSA and 4 MSSA) of S. aureus obtained from: University of Benin teaching hospital (UBTH), Benin, University of Nigeria teaching hospital (UNTH), Enugu, Jos University teaching hospital (JUTH), Jos and Cherith Diagnostic laboratory (CDL), Lagos, Nigeria were used. The MRSA and MSSA isolates were identified using standard microbiological methods which included colonial morphology, Gram's staining, biochemical and oxacillin screen agar tests (17, 18).

2.2 Plant samples collection and identification.

Fresh leaves of C. odorata were collected from their natural habitats in Igbinedion University, Okada environs, Edo State, Nigeria in the month of September, 2012. The plant part (leaves) selected in this study are commonly used in the locality for traditional medicine. The samples were identified and authenticated in the Department of Botany, University of Benin, Edo State and Forestry Research Institute of Nigeria (FRIN). The voucher specimen (Fhi no. 109890) was deposited in the herbarium.

2.3 Partitions of crude aqueous C. odorata extract

The crude aqueous leaf extract of C. odorata was prepared using the methods of Okigbo, et al., (19) and Nwinyi, et al., (20). The concentrated extract (300g) was dissolved in 800ml of distilled water. The solution was partitioned with chloroform to obtain two partitions, the aqueous (polar) and chloroform (non-polar) partitions. The partitions were evaporated to dryness using a rotary vacuum evaporator and weighed.

2.4 Screening of plant extracts for anti-MRSA activity

Each partition was then subjected to antibacterial assay on the MRSA and MSSA isolates to determine their activities. The control strain S. aureus NCIB 8588 was included (21, 22).The plant partitions were screened for anti-MRSA activity using the agar well diffusion technique. Mueller Hinton agar plates were prepared and with a sterile cork borer of 10 mm diameter, six wells were bored at equidistant after inoculation on each plate a standardized inoculum of 1-2 x 10^8 cfu/ml (compared with 0.5 McFarland standard) of each isolate. The 5^{th} and 6^{th} wells served as positive and negative controls respectively. Sterile distilled water in the case of soluble (aqueous) partition and dimethyl sulfoxide (DMSO) (Kermel) for insoluble (chloroform) partition of the plant served as the negative controls. Ciprofloxacin (1 mg/ml; Sigma-Aldrich, China) was used as the positive control. The aqueous partition was reconstituted with sterile distilled water (dimethyl sulfoxide was used for insoluble partition) and serially diluted using a double-fold dilution. A 0.2 ml of each prepared concentration of the plant partitions was aseptically introduced into wells 1- 4. The plates were left on the table for 40 minutes for pre-diffusion, followed by an overnight incubation at 37 ^{0}C. Zones of inhibition were measured in millimeters (23). The minimum inhibitory concentration (MIC) in mg/ml in this study was taken as the lowest concentration of each partition that inhibited the isolates (20).

2.5 Column chromatography of C. odorata chloroform partition

The chloroform partition (CP) was subjected to the fractionation process. It was fractionated by column chromatography (CC) using silica gel 60 (Qualikems). The CP was dissolved in ethyl acetate, poured on top of the silica gel column and eluted by stepwise elution with three mobile phases: ethyl acetate, chloroform and finally with methanol to obtain 142 fractions. Each mobile phase was poured continuously into the top of the column by the aid of a funnel. The bottom outlet of the column was opened. As the eluent (mobile phase) passed down the column, the components of the CP began to move down the column. The elutants (fractions) were collected in separate test-tubes (i.e. 10ml of elutant each) and kept for further analysis (24).

2.6 Thin Layer Chromatography (TLC) of C. odorata fractions.

Analytical TLC using commercially precoated silica gel 60 F_{254} papers (Merck, Germany) was performed. The solvent system used for the TLC analysis was chloroform-methanol in 4:1 ratio. The fractions obtained from column chromatography were spotted on the TLC papers equidistance apart and applied 1cm from the bottom of the papers. The spots were allowed to air-dry at ambient temperature for 30 minutes in order to evaporate the solvent from the spotted samples (fractions). The papers were inserted into the saturated tanks

which were covered and allowed to stand until the solvent had travelled to within 1cm away from the top edge of the papers. The papers were then removed and the solvent front marked. Spots on the TLC papers were visualized using iodine vapour (spray). After the spots were detected and marked, their retention factors (Rf value) were calculated and compared. The Rf values were calculated according to the formula:

$$\text{Rf value} = \frac{\text{Distance from the original point to the spot}}{\text{Distance from the original point to the front line}}$$

The fractions collected (from column chromatography) were added together on the basis of similar Rf results. The resultant fractions obtained after pooling were evaporated to dryness and weighed. Each fraction was subjected to antibacterial assay on the MRSA and MSSA isolates to determine their activities.

2.7 Statistical analysis

The data generated were presented as means and standard error. Analysis of Variance (ANOVA) and Duncan's Multiple Range (DMR) tests were used to establish significant differences where applicable. P values < 0.05 were regarded as significant and p values < 0.01as very significant. Statistical Package for Social Sciences (SPSS), version 20.0 software was used.

III. Results

Table 1 shows the percentage yields and antibacterial activities of C. odorata aqueous and chloroform partitions after 300g of the crude aqueous extract of the plant was partitioned with chloroform. The aqueous partition (AP) was 59.74% (179.22g) while the chloroform partition (CP) was 2.69% (8.07g). The total yield was 62.43% (187.29g). The activities of the aqueous and chloroform partitions were compared. The mean values of zones of inhibition at concentrations of 3.13- 25mg/ml were: 9.43 ±1.33mm and 16.61 ±0.93mm respectively. The minimum inhibitory concentration (MIC) was 12.5mg/ml for AP and 9.1% of the MRSA and MSSA isolates were inhibited. For the CP, 36.4% of the isolates were inhibited at that concentration. Furthermore, at 3.13 mg/ml, 9.1% of the isolates were inhibited by CP. There was a highly significant difference in activities between the chloroform partition (CP) and the aqueous partition (AP) ($p < 0.01$). There was no significant difference ($p > 0.05$) between the activity of MRSA and MSSA isolates in this study. Therefore, the chloroform partition was further analyzed.

The chloroform partition (6.8g) was subjected to silica gel column chromatography. The partition was eluted with mobile phases: ethyl acetate, chloroform and methanol at different ratios as shown in Table 2. A total of 142 fractions were obtained with different colours ranging from colourless, light yellow, yellow, green and brown. They are analyzed using thin layer chromatography (TLC).

The fractions with similar retention factors were added together which resulted into ten fractions labeled F1-F10 as shown in Table 3. The least mean retention factor was 0.43 (F8) and the highest was 0.93 (F10). The percentage yields of the TLC fractions of C. odorata after evaporation were: F1, 7.35% (0.5g); F2, F3, F5, F7 and F8, 8.82% (0.6g) and F4, F6 and F9, 10.29% (0.7g).

At a concentration of 2.0mg/ml, the fractions were tested for anti-MRSA activities. F2 and F3 gave the highest zones of inhibition of 22.5 $\pm$ 0.05mm and 22.5 $\pm$ 2.50mm respectively; followed by F8 (19.5 $\pm$ 4.50mm), then F1 (19.0 $\pm$ 2.0mm). Fraction F5 had the least activity with a mean value of 8.0 $\pm$ 8.0mm. There was no significant difference in activities between the fractions F1-F9 ($p > 0.05$) in this study. Therefore, in this present study, the fractions F1-F9 of C. odorata were observed to have more anti-MRSA activities than the aqueous (MIC of 12.5mg/ml) and chloroform (MIC of 3.13mg/ml) partitions of the plant.

Table 1. Yields and antibacterial activities of aqueous and chloroform partitions of C. odorata extract

			Diameter zone of inhibition(mm)		Percentage (%) of isolates with MIC mg/ml			
Partition	Yield (g)	Yield (%)	Mean value	Standard error	25	12.5	6.25	3.13
Aqueous (AP)	179.22	59.74	9.43[a]	± 1.33	54.50	9.10	–	–
Chloroform (CP)	8.07	2.69	16.61[b]	± 0.93	9.10	36.40	36.40	9.10
Key: Values with different letters are significantly different ($p < 0.01$) in activities.								
Negative control = Dimethyl sulfoxide/sterile, distilled water								

Table 2. Mobile phases used for column chromatography of the chloroform partition

Mobile phase	Ratio (%)	Volume (ml)	CC Fractions numbers	Colour observed
ethyl-acetate:chloroform	95 ; 5	100	1 – 25	brown
ethyl-acetate:chloroform	90 ; 10	100	26 – 42	dark green
ethyl-acetate:chloroform	85 ; 15	50	43 – 48	green
ethyl-acetate:chloroform	80 ; 20	50	49 – 54	green
ethyl-acetate:chloroform	75 ; 25	50	55 – 64	yellow
ethyl-acetate:chloroform	70 ;30	50	65 – 72	yellow
ethyl-acetate:chloroform	50 ; 50	50	73 – 80	yellow

ethyl-acetate:chloroform	40 ; 60	20	81 – 83	yellow
ethyl-acetate:chloroform	30 ; 70	20	ND	ND
ethyl-acetate:chloroform	20 ; 80	20	84 – 87	yellow
ethyl-acetate:chloroform	10 ; 90	20	88 – 90	yellow
chloroform	100	20	91 – 94	light yellow
chloroform: methanol	90 ;10	20	95 – 98	light yellow
chloroform: methanol	80 ; 20	20	99 – 102	light yellow
chloroform:methanol	70 ; 30	20	103 - 106	colourless
chloroform:methanol	60 ; 40	20	107 - 110	colourless
chloroform:methanol	50 ; 50	20	111 - 114	colourless
chloroform:methanol	40 ; 60	20	115 - 119	colourless
chloroform:methanol	30 ; 70	20	120 - 123	colourless
chloroform:methanol	20 ; 80	20	124 - 127	colourless
chloroform:methanol	10 ; 90	20	128 - 132	colourless
methanol	100	50	133 - 142	colourless
Key: ND = not done; Weight of chloroform partition used = 6.8 g; CC = column chromatography				

Table 3. Yields and antibacterial activities of TLC fractions of C. odorata

CC Fractions numbers	Mean Rf value	TLC fraction label	Yield (g)	Yield (%)	Mean value	Standard error
1 – 5	0.86	F1	0.5	7.35	19.0	± 2.00
6 -12	0.66	F2	0.6	8.82	22.5	± 0.50
13 -35	0.56	F3	0.6	8.82	22.5	± 2.50
36 -65	0.59	F4	0.7	10.29	10.5	± 10.5
66 - 67	0.85	F5	0.6	8.82	8.0	± 8.00
68 - 88	0.82	F6	0.7	10.29	16.5	± 0.50
89 - 100	0.74	F7	0.6	8.82	10.0	± 10.0
101 - 108	0.43	F8	0.6	8.82	19.5	± 4.5
109 - 120	0.49	F9	0.7	10.29	11.0	± 11.0
121 -142	0.93	F10	ND	ND	ND	ND
Key: Rf = Retention factor; CC = column chromatography, Negative control = Dimethyl sulphoxide; Positive control = Ciproxacillin (1 mg/ml)						

IV. Discussion

Comparative anti-MRSA activities of seven selected Nigerian medicinal plants have been previously determined (6). The solvents used for the plants extraction were: hexane, ethanol and water. The researchers reported that A. conyzoides, B. pinnatum, P. pellucida and O. gratissimum showed no anti-MRSA activities while C. odorata, P. guineense and C. pilosa showed activities. In their study, they concluded that the crude aqueous and ethanolic extracts of C. odorata were considered the most efficacious of the seven selected medicinal plants.

In this study, the crude aqueous extract of C. odorata was partitioned (using chloroform) and fractionated (via column and thin layer chromatographic techniques) into fractions to further determine the plant's anti-MRSA activity. Two C. odorata partitions (aqueous partition (AP) and chloroform partition (CP)) were obtained. Although the percentage yield of the AP was higher than the CP, the anti-MRSA activity of the CP was higher. The minimum inhibitory concentration (MIC) of the latter was 3.13mg/ml and the mean value for the zone of inhibition was 16.61±0.93mm.There was a highly significant difference ($p < 0.01$) in the activities of the two fractions (AP and CP). This result disagrees with the finding of Jualang, et al., (25) who reported that chloroform extract (CE) at 100 mg/ml showed a weak zone of inhibition of 7.0±0.00mm against S. aureus. However, it agrees with the finding of Sukanya, et al., (10) who reported that the CE of C. odorata showed an MIC value of 4.0 mg/ml against S. aureus. There was no significant difference in the activities of MRSA and MSSA (methicillin susceptible S. aureus) isolates used in this study.

Because of the higher anti-MRSA activity of the chloroform partition (CP) of C. odorata in this study, it was further analyzed. It was fractionated using column and thin layer chromatographic techniques. One hundred and forty-two chloroform fractions obtained after column chromatography had varieties of colours which were: colourless, light yellow, yellow, green and brown. The fractions were subjected to thin layer chromatography (TLC) and ten fractions with retention factor (Rf) values from 0.43 to 0.93 (indicating the presence of different groups of phytochemicals in the plant) were obtained. This range of Rf values was also reported by other researchers who stated that Rf values ranged between 0.3 to 0.9 indicated the presence of terpenes, phenolic acids and flavonoids (26, 27).TLC profiling of plant extracts has resulted in directing towards the presence of a number of phytochemicals. Different Rf values of compounds (plant fractions) reflect about their polarity. A compound/fraction showing high Rf value in a solvent system have low polarity and those with a less Rf value have high polarity. Mixture of solvents with variable polarity in different ratio can be used for the separation of pure compounds from plant extracts (27).

Flavonoids and phenolic acids are the most important groups of secondary metabolites and bioactive compounds in plants. They are also a kind of natural products and antioxidant substances capable of scavenging free superoxide radicals, anti-aging and reducing the risk of cancer. It was found that flavonoids reduce blood-lipid and glucose of humans. They constitute a wide range of substances that play important roles in protecting biological systems against the harmful effects of oxidative processes on macromolecules such as carbohydrates, proteins, lipids and DNA (28). Flavonoids are known to play a protective role against microbial invasion in plants that synthesize these polyphenols. This protective role involves the presence of flavonoids in plants as constitutive agents as well as their accumulation as phyto-alexins in response to microbial attack. It is not surprising therefore, that plants rich in flavonoids have been used in many years in traditional medicine to treat infectious diseases (28, 29). Their activity is probably due to their ability to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (30, 31).

Varied biological activities of phenolic acids have been reported such as: increase bile secretion, reduce blood cholesterol and lipid levels and antimicrobial activity against some strains of bacteria such as S. aureus (30). The mechanisms thought to be responsible for phenolic acids toxicity to microorganisms include enzyme inhibition, substrate deprivation and complex with cell wall (30, 31).

As a broad group, terpenes exhibit a range of toxicity from deadly to entirely edible and this is in keeping with their broad range of ecological roles. These roles include: antimicrobial properties and a range of properties that attract symbiotes for the purposes of pollination, seed dispersal and secondary protective roles. These latter roles include the provision of airborne chemical signals and, flavour and taste (32). Terpenes or terpenoids are active against bacteria, fungi, viruses and protozoa. It was reported that 60% of essential oil derivatives were inhibitory to fungi while 30% to bacteria. The triterpenoid betulinic acid is just one of several terpenoids which have been shown to inhibit HIV. The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds (30, 31).

The highest antibacterial activities were observed scents in TLC fractions F2 and F3 of C. odorata. This agrees with the finding of Jualang, et al., (25) who also reported that the fraction F2 of the ten fractions obtained in their study on C. odorata had the highest activity against S. aureus. However, the activities observed in fractions F2 and F3 in this study were higher than that reported by Jualang, et al., (25). Therefore in this study, the fractions F1–F9 of C. odorata were observed to have more anti-MRSA activities than the aqueous (MIC of 12.5mg/ml) and chloroform (MIC of 3.13mg/ml) partitions of the plant. Furthermore with reference to the work of Okwu, et al., (6), the fractions obtained in this study had more anti-MRSA activities than the crude aqueous, hexane and ethanolic extracts of C. odorata. This could be due to the fact that the fractions consist of pure compounds while the crude extracts and partitions of the plant contained various types of compounds.

V. Conclusion

The present study reveals that C. odorata is a potential and promising plant that should be exploited for the management of diseases caused by MRSA (and perhaps some other drug-resistant microorganisms) because its fractions have more anti-MRSA activity than its crude extracts and partitions. However, further research is necessary to determine the identity and full spectrum of efficacy of the anti-MRSA fractions (compounds) from within this plant.

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