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PHYTOCHEMICAL AND ANTIMICROBIAL SCREENING OF THE PARTITIONED-SOLUBLE PORTIONS OF THE DE-PIGMENTED METHANOLIC EXTRACT OF *TRIDAX PROCUMBENS* LINN (WHOLE PLANT)

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Abstract
The five partitioned-soluble portions of the earlier reported antibacterial active de-pigmented crude methanolic extract of T. procumbens (Mp-C) was further screened for their phytochemicals and antimicrobial efficacy. Phytochemical screening of the soluble portions using standard methods revealed that the butanol-soluble portion (Mp-Cb) like the crude extract (Mp-C) showed the strong presence of saponins, flavonoids, sapogenins and alkaloids. The in-vitro antibacterial assay of the test compounds using the agar-diffusion method revealed that the ethyl acetate- (Mp-Ce), butanol- (Mp-Cb) and chloroform- (Mp-Cc) soluble portions at 50 mg/ml exhibited significant inhibitory broad spectrum activities than Mp-C better than that exhibited by tetracycline at 1 mg/ml against some of the test bacterial strains. In-vitro antifungal assay of the test compounds using also the agar-diffusion method revealed that all the portions expressed no significant activity, while Mp-C exhibited weak antifungal activity at 50 mg/ml in comparison with ketoconazole at 1 mg/ml against the test fungal strains. The minimum inhibitory (MIC), minimum bactericidal (MBC) concentrations and MBC/MIC ratios of the active portions ranged from 6.25-12.5 mg/ml, 6.25-25 mg/ml and 1.00–2.00, respectively, while the MIC, minimum fungicidal (MFC) concentrations and MFC/MIC ratio of Mp-C was 25 mg/ml, 50 mg/ml and 2.00, respectively. The above findings suggest that the partitioned-soluble portions of the de-pigmented methanol extract of T. procumbens might be a veritable source of antibacterial, rather than antifungal substances for the treatment of diarrhea, typhoid fever, urinary and gastrointestinal infections.
Keywords: *Tridax procumbens*, methanol, de-pigmented, phytochemicals, antibacterial, antifung

INTRODUCTION

Tridax procumbens L. (Family: Asteraceae) is a specie of flowering plant that is commonly found in the tropical and subtropical region. It is locally known as Igbalode (Yoruba), Gogomasi (Hausa) and wild daisy (common name). It is a perennial herb with many branches, leaves are toothed and arrowhead-shaped, flowers are daisy-like yellow-centered white and fruit is a black seed covered with stiff hairs (Holms *et al.*, 1997; Sueseela *et al.*, 2002; Mann *et al.*, 2003). The crude methanolic extract of the plant (whole) on removal of pigments (de-pigmentation) at 100 mg/ml and its purified fractions from preparative thin layer chromatography at 50 mg/ml, reportedly exhibited significant inhibitory activity than the non-depigmented crude methanolic extract at 100 mg/ml against some bacterial strains (Fadipe *et al.*, 2011).

The present study therefore, aims at further partitioning of the active crude methanolic extract (Mp-C) between water and various organic solvents. The obtained partitioned-soluble portions were screened for the presence of various secondary metabolites and the antimicrobial efficacy against selected bacteria and fungi determined, in comparison with the crude de-pigmented extract (Mp-C) and a standard antibacterial and antifungal agent. Partitioning between solvents is an adequate approach for the preliminary separation of complex plant matrices, because the procedure permits discrimination of activities between the polar and non-polar fractions (Mahlke *et al.*, 2009).

MATERIALS AND METHODS

Collection and Identification of Plant Material

Tridax procumbens (whole plant) was collected within the premises of Federal University of Technology, Minna, Niger State, Nigeria, in the month of June, 2010. The plant was duly identified and deposited at the Herbarium, Department of Biological Sciences, Faculty of Science, Ahmadu Bello University, Samaru, Zaria, Nigeria.

Extraction Procedures

Five hundred grams (500 g) of air-dried *T. procumbens* (whole plant) was defatted with 1.5 L of petroleum ether (60-80°C) using the continuous extraction method. The defatted marc was air-dried and extracted with 2.5 L of methanol. The resulting solution was concentrated in vacuo using a rotavapour, dried over a water bath and the extract labeled 'Mp'.

De-pigmentation of Crude Methanol Extract (Mp)

The method of Hostettmann *et al.* (1998) was adopted. 50 g of methanol extract of *T. procumbens* (Mp) was solubilized in 800 ml of methanol and mixed thoroughly with 250 g of activated charcoal until a right consistency was achieved. This was tightly sealed and kept aside for 72 h. The mixture was filtered and the residue washed severally with methanol to ensure a pigment-free extract. Filtrate was concentrated in-vacuo, dried and labelled 'Mp-C'.

Partitioning of Crude De-pigmented Methanol Extract (Mp-C)

Thirty-five grams (35 g) of Mp-C was suspended in 300 ml of distilled water, shaken vigorously and the mixture allowed to stand for 2 h after which it was filtered and the filtrate in a separatory funnel, partitioned with 100 ml x 3 portions of petroleum ether. The organic phase was removed, concentrated in vacuo, dried, weighed and coded petroleum ether-soluble portion of partitioned methanolic extract of *T. procumbens* (Mp-Cp; 0.27%). The residual water-soluble portion was again successively and exhaustively partitioned with 100 ml x 4 portions of chloroform, 100 ml x 5 portions of ethyl acetate and 100 ml x 7 portions of n-butanol respectively. The resulting organic portions were concentrated, dried, weighed and coded $CHCl_3$-soluble (Mp-Cc; 3.78%), EtOAc-soluble (Mp-Ce; 6.44%) and BuOH-soluble (Mp-Cb; 10.4%) portions of partitioned methanolic extract of *T. procumbens*, respectively. The residual aqueous portion was concentrated, dried, weighed and coded (Mp-Cr, 64.9%).

Phytochemical Screening of the Partitioned-Soluble Portions

The partitioned-soluble portions (Mp-Cp, Mp-Cc, Mp-Ce, Mp-Cb and Mp-Cr) in comparison with its crude de-pigmented methanolic extract (Mp-C) were screened for the presence of various secondary metabolites using standard methods (Sofowora, 1993; Evans, 1996).

Determination of *In-vitro* Antimicrobial Efficacy of the Soluble Portions

Microbial Strains: The five partitioned-soluble portions obtained from the crude de-pigmented methanolic extractin comparison with the crude de-pigmented methanolic extract (Mp-C)were tested against six bacterial strains (two *Gram* positive: *Bacillus subtilis*and *Staphylococcus aureus*; four *Gram* negative: *Escherichia coli, Klebsiella pneumonia* and *Salmonella typhi*-a clinical isolate) and five fungal strains (three filamentous: *Aspergillus flavus, Aspergillus niger* and *Penicillium citrinium;* two yeasts: *Saccharomyces cereviseae* and *Candida albicans*-a clinical isolate). All bacterial and fungal stock cultures were obtained from the Department of Microbiology, Federal University of Technology, Minna, Niger state.

Preparation of Inocula: The viability test for each organism was carried out by resuscitating the bacterial strains on nutrient agar medium (NA, MHA) and incubated at 4°C for 24 h. The filamentous fungal strains were maintained at 28°C on potato dextrose agar (PDA, Oxoid) for 3 days, while the yeasts were maintained at 37°C on Sabouraud dextrose agar (SDA, Oxoid) for 24 h. The stock cultures were diluted with fresh MHA or PDA or SDA to achieve 1 x 10^6 cfu/ml (bacteria) and 1 x 10^6 spore/ml (fungi), respectively (Duraipandiyan *et al.*, 2006).

Antibacterial Susceptibility Testing: The agar-well diffusion method was used to test the antibacterial efficacy of the test compounds (Perez *et al.*, 1990; Okeke *et al.*, 2001). One hundred microliters (100 µl) of standardized inoculum of each test bacterium was evenly spread onto the surface of freshly prepared sterile MHA plates using sterile cotton swab. 6 mm wells were bored into the solidified agar using a sterile cork borer at equidistant. Subsequently, 100 µl of each reconstituted soluble portionand Mp-C (50 mg/ml) was aseptically introduced in triplicate wells of the agar plates with the aid of a Pasteur pipette, while, methanol and 1 mg/ml tetracycline(La tetra-250, Meeure Nig. Ltd., Lagos)were used as negative and positive controls, respectively. Plates were allowed to stand for 1 h for diffusion to take place and then incubated aerobically at 37°C for 24 h. The antibacterial activity of test compounds were evaluated by measuring the inhibition zone diameter (IZD) around the wells to the nearest millimeter and recorded as the mean IZD. Plates for extract sterility control (ESC), organism viability control (OVC) and medium sterility control (MSC) were also prepared alongside and also incubated aerobically at 37°C for 24 h.

Antifungal Susceptibility Testing: The agar-well diffusion method was also used to determine the antifungal efficacy of the test compounds as shown above. One hundred microliters (100 µL) of standardized inoculum of each filamentous and yeasts test fungi was evenly spread onto the surface of freshly prepared sterilized PDA and SDA plates respectively. 6 mm wells were bored into the solidified agar for each and treated as for antibacterial testing. Methanol and 1 mg/ml ketoconazole (Janssen Pharmaceutical N. V., Belgium) were used as negative and positive controls respectively. Plates were incubated at 28°C and IZD measured after 48 h for filamentous fungi, while plates were incubated at 37°C and IZD measured after 24 h for yeasts.

Analysis of Results

Experiments were carried out in triplicates and results analyzed for statistical significance. Comparisons between groups were performed using two-way analysis of variance (ANOVA) on statistical software package-Statistical Package for Social Sciences (SPSS 15.0 for Windows, 2006 version) with Ryan-Einot-Gabriel-Welsch F Post hoc tests for separation of means. Differences were considered significant, if p 0.05. A plant extract is considered 'active', when it has an inhibition zone diameter (IZD) of © 14 mm (Mothana and Linderquist, 2005).

Determination of Minimum Inhibitory Concentration (MIC)

MIC for all antibacterial active test compounds was determined using the tube dilution method.A two-fold serial dilution of each active test compound was added to 1 ml sterile molten culture (nutrient broth, NA, Oxoid for bacteria; potato dextrose broth, PDB, Oxoid for fungi) in a series of test tubes to achieve a decreasing concentration range of 50, 25, 12.5, 6.25, 3.125 mg/ml. Tubes were inoculated with a loop full of standardized inocula (1 x 10^6 cfu/ml and 1 x 10^6 spore/ml for bacteria and fungi respectively). NA and PDB containing a loop full of the inocula only, served as positive controls for bacteria and fungi respectively. Tubes were incubated at 37°C for 24 h (bacteria and yeasts) and 28°C for 48 h (filamentous fungi). The MIC was considered as the lowest concentration showing no detectable growth/no turbidity/clear zone of inhibition (Perez *et al.*, 1990).

Determination of Minimum Bacteriocidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

A loop full of culture was collected from each set of tubes that did not show clear zones of inhibition from the MIC tubes above and sub-cultured onto freshly prepared MHA/PDA/SDA plates for bacteria, filamentous fungi and yeasts, respectively. Inoculated plates were incubated at 37°C for 24 h (bacteria and yeasts) and 28°C for 48 h (filamentous fungi). The concentration at which no visible growth was observed after incubation was taken as the MBC (bacteria) and MFC (fungi).

MBC/MIC and MFC/MIC Ratios of the Active Test Compounds

The MBC/MIC and MFC/MIC ratios of the active test compounds were calculated using the method of Agnese *et al.* (2001).

RESULTS AND DISCUSSION

Preliminary phytochemical screening of the partitioned-soluble portions of Mp-C (Table 1) showed that the butanol-soluble portion (Mp-Cb) revealed the presence of almost same metabolites as the crude extract (Mp-C), an indication that most of the constituents present in Mp-C were extracted into the butanol-soluble portion. Strong presence of metabolites, such as saponins, carbohydrates, flavonoids and alkaloids also indicates that the secondary metabolites present in the de-pigmented methanol extract and its portions are mostly of mid-polar/polar composition. Different solvents have been demonstrated to have the ab[illegible]

metabolites which depends on their polarity and solubility in the solvents (Marjorie, 1999). Antibacterial assay of the partitioned-soluble portions of Mp-C (Table 2) revealed that the chloroform (Mp-Cc), ethyl acetate (Mp-Ce) and butanol (Mp-Cb) soluble portions significantly inhibited the growth of all bacteria used in this study. The chloroform portion (Mp-Cc) revealed an inhibition zone diameter (IZD) that was similar to that exhibited by tetracycline against *E. coli*. The antibacterial activity of some of the portions was better than that of tetracycline against *Gram* positive *B. subtilis*, and *S. aureus*, *Gram* negative *P. aeruginosa* and *K. pneumonia*. This is noteworthy, because crude plant preparations have reportedly exhibited lower antimicrobial activity than pure antibiotics (Iroegbu and Nkere, 2005). Some of the soluble portions exhibited broad spectrum activity that was better than that exhibited by Mp-C, although, the portions revealed the presence of same constituents as Mp-C. This may be attributed to the presence of some constituents which may b acting synergistically with one another or with othe constituents in the portions(Doughari and Obidah, 2008) o may be the active constituents which are usually present ir trace or dilute amount become more concentrated with partitioning and therefore enhanced activity is observed (Ndip *et al*., 2009). The partitioned-soluble portions expressed practically no antifungal activity against all the fungal strains, while Mp-C was only slightly active against the fungal strains in comparison with ketoconazole (Table 3). This shows that the constituents present in the partitioned-soluble portions (Table 1) could not inhibit the growth of any of the test fungal strains at that concentration. This could be as a result of may be the antifungal components are present in trace or dilute amount (Dall'Agnol *et al*., 2003) or probably antifungal activity of the constituents could be enhanced at increased concentration.

Table 1: Secondary metabolites in the crude de-pigmented methanolic extract of *T. procumbens* (Mp-C) and its partitioned-soluble portions

Phytochemicals	Mp-C	Mp-Cp	Mp-Cc	Mp-Ce	Mp-Cb	Mp-Cr
Carbohydrates	+++		-	+	+++	++
Tannins	++	-	-	+	++	+
Phlobatannins	-	-	-	-	-	-
Saponins	+++	-	-	+	+++	+
Flavonoids	+++	-	++	+++	+++	-
Cardiac glycosides	+++	-	+	++	+++	+
Anthraquinones	+++	-	+	++	++	-
Coumarins	++	+	+++	++	++	-
Tetraterpenoids	-	-	-	-	-	-
Alkaloids	+++	-	+	+++	+++	-

+ = low concentration; ++ = moderate concentration;
+++ = high concentration; - = absent

Table 2: Antibacterial activity of crude methanolic extract of *T. procumbens* (Mp-C) and its partitioned -soluble portions at 50 mg/ml against som e bacterial strains

Test compound	Inhibition zone diameter of test organisms (mm)*					
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*K. pneumonia*	*S. typhi*
Mp-C	-	^{g}±0.31	11.5^{ef}±0.32	10.9^{fg}±0.25	10.3^{gh}±0.46	9.1^{ij}±0.31
Mp-Cp		5.93^{lm}±0.15	-	-	8.37^{no}±0.25	12.1^{ef}±0.12
Mp-Cc	16.0^{bc}± 0.20	17.9^{ab}±0.25	22.1^{a}±0.26	19.1^{bc}± 0.10	18.0^{cd}±0.31	16.6^{cd}± 0.26
Mp-Ce	21.0^{a}±0.20	17.0^{cd}±0.06	19.2^{cd}±0.21	23.0^{a}± 0.00	16.2^{de}±1.15	-
Mp-Cb	18.9^{ab}±0.20	22.0^{a}±0.25	16.0^{de}±0.15	15.0^{ef}±0.06	12.1^{ef}±0.25	18.1^{bc}±0.15
Mp-Cr	6.2^{kl}±0.26	5.0^{lm}±0.15	10.0^{fg}±0.20	5.23^{hi}±0.15	-	7.57^{fg}±0.35
Tetracycline (1 mg/ml)	16.0^{bc}±0.25	15.5^{de}± 0.25	21.2^{ab}±0.30	13.0^{de}±0.25	16.5^{de}±0.15	24.6^{a}±0.25

- = no measurable zone of inhibition; * = mean values of three replicates with standard error shown as ±; Mean values on the same column/row with same superscript (letters/numbers) are not significantly different from each other ($p > 0.05$) while those with different superscript (letters/numbers) are significantly different from each other ($p < 0.05$), respectively.

Table 3: Antifungal activity of crude methanolic extract of *T. procumbens* (Mp-C) and its partitioned -soluble portions at 50 mg/ml against some fungal strains

Test compound	Inhibition zone diameter of test organisms (mm)*				
	A. Flavus	*A. niger*	*P. citrinium*	*C. albicans*	*S. cereviseae*
Mp-C	$14.0^{b3}\pm0.25$	$15.2^{b2}\pm0.26$	$11.0^{b4}\pm0.30$	$15.8^{b1}\pm0.21$	$10.0^{b5}\pm0.21$
Mp-Cp	$6.03^{c2}\pm0.28$	-	$8.0^{c1}\pm0.20$	-	-
Mp-Cc	$8.1^{d3}\pm0.21$	$11.0^{c2}\pm0.21$	-	$12.0^{c1}\pm0.20$	-
Mp-Ce	-	-	-	-	$7.50^{c1}\pm0.46$
Mp-Cb	$10.8^{c1}\pm0.21$	-	-	$10.1^{d2}\pm0.15$	-
Mp-Cr	-	-	-	-	$6.93^{d1}\pm0.15$
Ketoconazole (1 mg/ml)	$20.9^{a2}\pm0.21$	$23.1^{a1}\pm0.17$	$13.9^{a4}\pm0.20$	$20.1^{a3}\pm0.25$	$12.7^{a5}\pm0.25$

- = no measurable zone of inhibition; * = mean values of three replicates with standard error shown as ±; Mean values on the same column/row with same superscript (letters/numbers) are not significantly different from each other ($p > 0.05$) while those with different superscript (letters/numbers) are significantly different from each other ($p < 0.05$), respectively.

The MIC, MBC and MBC/MIC values of the active partitioned -soluble portions and tetracycline against the test bacterial strains ranged from 6.25 -12.5 mg/ml, 6.25 -25 mg/ml and 1.00 -2.00; 6.25 -12.5 mg/ml, 3.125 -12.5 mg/ml and 0.50 -1.00, respectively (Table 4). Against the fu ngal strains, MIC, MBC and MBC/MIC values for the active crude extract and ketoconazole was 25 mg/ml, 50 mg/ml and 2.00; 6.25 mg/ml; 3.125 - 6.25 mg/ml and 0.50 -1.00, respectively (Table 5). The lower MIC and MBC values of the soluble portions against bacter ial strains over that of the crude methanolic extract against fungal strains, is an indication of the antibacterial efficacy of the plant (Fabry *et al.*, 1998). MBC/MIC and MFC/MIC ratios greater than 1 is an indication that the antibacterial effects produc ed by the active partitioned -soluble portions and antifungal effect produced by the crude de -pigmented methanolic extract were bacteriostatic and fungi static in action, respectively (Agnese *et al.*, 2001).

Table 4: MIC; MBC and MBC/MIC values of active p ortions of *T. procumbens* against some bacterial strains in comparison with tetracycline

Test Compound	MIC (mg/ml); MBC (mg/ml); *MBC/MIC values of test compounds against test organisms					
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*K. pneumonia*	*S. typhi*
Mp-Cc	12.5; 25; 2.00	12.5 ;25; 2.00	6.25; 6.25; 1.00	12.5; 12.5; 1.00	12.5; 25; 2.00	12.5; 25; 2.00
Mp-Ce	6.25; 6.25; 1.00	12.5; 25; 2.00	6.25; 6.25; 1.00	6.25; 6.25; 1.00	12.5; 25; 2.00	ND
Mp-Cb	12.5; 12.5; 1.00	6.25; 6.25; 1.00	12.5; 25; 2.00	12.5; 25; 2.00	ND	12.5; 12.5; 1.00
Tetracycline	12.5; 12.5; 1.00	12.5;12.5; 1.00	6.25; 3.125; 1.00	ND	12.5; 12.5; 1.00	6.25; 3.125; 0.50

ND = Not determined; *MBC/MIC ratios; **> 1** = Bacteriostatic effect; **< 1** = Bacteriocidal effect.

CONCLUSION

The partitioning of the antibacterial active de-pigmented methanol extract of *Tridax procumbens* showed that the portions displayed significant antibacterial activity better than the crude extract. The antibacterial components of the crude extract were better extracted into the chloroform-, ethyl acetate- and butanol-soluble portions. The portions displayed no significant antifungal activity, while the crude extract exhibited weak antifungal activity in comparison to the standard drug. This shows that the partitioning of an extract could make a medicinal plant an effective source of antibacterial substances. Further work will aim at isolation and characterization of the antibacterial active constituents.

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