PHYTOCHEMICAL AND *IN VITRO* ANTIMICROBIAL SCREENING OF THE ETHANOLIC EXTRACT OF *Theobromacacao* SEEDS AND ITS FRACTIONS

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Abstract
Phytochemical screening of the crude ethanolic extract of T. cacao seeds and its partitioned-soluble portions revealed the presence of carbohydrates, flavonoids, saponins, tannins, alkaloids, steroidal nucleus and cardiac glycosides. The extract and its soluble portions were evaluated against sixteen pathogenic organisms at 4,000µg/ml using agardilution method. The ethanolic extract exhibited bacteriostatic effect on only Escherichia coli (ATCC 9637), while petroleum ether extract and the partitioned soluble portions of the ethanolic extract had no inhibitory effect on any of the test organisms at the same concentration. Further purification of the active extract using column chromatography gave rise to four major fractions that revealed varying number of spots in different solvent media. These spots presented different colors under sunlight, UV light and iodine crystals. Antibacterial assayof these fractions against E. coli, showed that three of the fractions also inhibited the growth of the organism at 4,000µg/ml. The minimum inhibitory concentration (MIC)and minimum bacteriocidal concentration (MBC) of the active ethanolic extract was4000µg/ml for both, while that of the active fractions ranged from 3250-3500µg/ml and 3250-3500µg/ml respectively.This shows that the crude ethanolic extract of the seeds of T. cacao and its fractions could be a potential source of antimicrobial agents.

Keywords: Column chromatography,ethanolic extract, *Escherichia coli,*fractions,

Introduction
Theobroma cacao Linn (Family: *Sterculiaceae*, formerly, *Malvaceae*) is a tropical evergreen small tree. It has perennial leaves and small reddish flowers with pink calyx. Fruits (cocoa pods) are yellowish-red and of variable sizes. They are made up of numerous seeds (cocoa beans), which are fleshy and embedded in a white pulp. These seeds are the sole source of cocoa powder and butter which are important ingredients used in chocolate, pharmaceutical and cosmetic industries (Silva *et al*., 2009). Fermented roasted seeds are very rich in fats (Henderson *et al.,* 2009). Cocoa seeds have been reported to possess anti-cariogenic activity (Ooshima*et al*., 2000), anti-tumor activity (Preza*et al.,* 2010), anti-oxidant activity (Othman *et al*., 2007; Bubonja-Sonje*et al.,* 2011) and anti-listerial activity (Bubonja-Sonje*et al.,* 2011) as a result of the presence of flavonols and procyanidins (flavonoids) in the seeds (Kenny *et al*., 2004). Theantibacterial activity of the ethanolic extract of the seed bark against penicillin G resistant strain of *Staphylococcus aureus*has been reported (Perez and Anesini, 1994;Smullen*et al.,* 2007; Bubonja-Sonje*et al.,* 2011).

In view of the wide range of the medicinal values of the seeds of *T. cacao,*it is of interest to study the antimicrobial efficacy of extracts/portions of these seeds against a wider range of organisms. This work, therefore screens the ethanolic extract of the seeds of *T. cacao*, its partitioned-soluble portions and its fractions phytochemically and their *in vitro* antimicrobial properties against a wide range of selected microbes using standard methods.This becomes pertinent in order to find natural product alternatives that will help prevent the widespread of microbial infection.

Materials and Method

Collection of plant material

Fresh pods of *T. cacao* were collected from a farm in Ifon, Orolu Local Government Area of Osun state, Nigeria in the month of August, 2010. The plant was identified and authenticated by MallamGallahof the herbarium section, Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria and a voucher specimen made and deposited. The seeds were then separated from the pods, air-dried at room temperature and pulverized.

Extraction of plant material

Five hundred grams (500g) of pulverized air-dried seeds of *T. cacao* was macerated and extracted exhaustively with 96% ethanol for a week at room temperature. The resulting mixture was filtered, concentrated in vacuo using a rotatory evaporator and evaporated to dryness over a water bath at 70^{o}C to yield a dark brown gummyextract coded 'TcE'(% yield, 8.26). The extract was further macerated and exhaustively defatted with petroleum ether for 3 days at room temperature. Extract was treated as for TcEto yield a dark brown oily mass coded 'TcP' (% yield, 5.3)

Phytochemical screening of the crude extracts

The concentrated extracts, TcP and TcE were screenedfor the presence of various secondary metabolites using standard methods (Sofowora, 1993; Evans, 1996)

Partitioning of crude ethanolic extract (TcE)

Twenty grams (20g) of TcE was suspended in 200ml of distilled water, shaken vigorously and the mixture allowed to stand for 2h after which it was filtered and the filtrate in a separatory funnel, partitioned with 100ml x 5 portions of chloroform. The organic phase was removed, concentrated in vacuo, dried, weighed and coded $CHCl_3$-soluble portion of partitioned ethanolic extract of *T.cacao* (TcEc;golden brown mass; % yield, 13.5). The residual water-soluble portion was again successively and exhaustively partitioned with 100ml x 5 portion of ethyl acetate and 100ml x 6 portions of n-butanol, respectively. The resulting organic portions were concentrated, dried, weighed and coded EtOAc-soluble (TcEe; dark brown mass; % yield, 21.5) and BuOH-soluble (TcEb; brownish gummy mass; % yield, 31) portions of partitionedethanolic extract of *T. cacao,* respectively. The residual aqueous portion was concentrated, dried, weighed and coded (TcEr; light brown gummy mass;% yield, 28).

Phytochemical screening of the partitioned-soluble portions

All partitioned-soluble portions (TcEc, TcEe, TcEb and TcEr) werealso screened for the presence of various secondary metabolites (Sofowora, 1993; Evans, 1996).

Source of microorganisms

The extracts/portions obtained from the seeds of *T. cacao*were tested against sixteen isolates.The bacterial strains used were:*Bacillus subtilis*(clinical strain),*Staphylococcus spp.* (clinical strain A and B), *Staphylococcus aureus* (clinical strain and ATCC 13709), *Staphylococcus epidermidis* (clinical strain)*Escherichia coli* (clinical strain *and* ATCC 9637), *Pseudomonas aeruginosa* (clinical strain and ATCC 27853), *Proteus spp.* (clinical strain), *Salmonella gallinarium*(clinical strain), *Salmonella paratyphi* C (clinical strain) and *Salmonella typhi* (clinical strain), while thefungal (yeast) strains were: *Candida albicans* (clinical strain and ATCC 10231). All bacterial and fungal stock cultures were obtained from Microbiology unit, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Kaduna State, Nigeria and National Institute for Pharmaceutical Research and Drug development, Idu, Abuja, Nigeria.

Preparation of inoculums

The viability test for each organism was carried out by resuscitating each microorganism on nutrient agar medium (NA, MHA) and incubating at 4^{o}C for 24h. The stock cultures were diluted with fresh MHA to achieve 1x10^{6}cfu/ml(Duraipandiyan*et al.*, 2006). A loopful of the standard culture was used for the antimicrobial assay.

Antimicrobial susceptibility testingof crude extracts/portions

The agar dilution method was used to test the antibacterial/antifungal efficacy of the crude extracts/portions/standard drug (Babayi*et al.*, 2007). 400mg of each extract/portion/drug was reconstituted in 1ml tween-80 after which 4ml of sterile distilled water was added. 1ml of each reconstituted extract/portion/drug was then transferred to sterile Petri dishes containing 19ml of MHA. Plates were prepared in duplicates and allowed to set at room temperature. A loopful of standardized bacterial/yeast culture was streaked unto each solidified agar plate. Plates for standard (positive) control, extract sterility control (ESC), organism viability control (OVC) and medium sterility control (MSC) were also prepared alongside. All plates were incubated aerobically at 37^{o}C for 24h.

Determination of minimum inhibitory concentration (MIC)

The NIPRD protocol of 2006 was adopted. Briefly, 1ml of the active extract solution (TcE) at 4,000µg/ml was added to 19ml of sterile Mueller Hinton broth and subsequently transferred to make solution of varying concentrations of 4,000, 3750, 3500, 3250, 3000, 2750, 2500, 2250 and 2000 µg/ml respectively. A loopful of the standardized *E. coli* (ATCC 9637) was inoculated into the broth containing different concentration of the extract and incubated at 37^{o}C for 24h. The test tubewith the least concentration of the extract at which no detectable growth was observed was considered as the MIC.

Determination of minimum bacteriocidal concentration (MBC)

A loopful of culture was collected from the tubeshowing no detectable growth from the MIC tubes above and sub-cultured onto freshly prepared MHA plates.Inoculated plates were incubated at 37^{o}C for 24h. The least concentration at which no detectable growth was observed after incubation was taken as the MBC.

Fractionation of crude ethanolicic extract (TcE)

Ten grams (10g) of 'TcE' was fractionated using column chromatography. The column was packed with silica gel (60-120 mesh) and chloroform using the wet method.Varying proportions of increasing polarity of chloroform-methanol was used as the mobile phase. Identical fractions were pooled using TLC and concentrated under vacuum. Fractions obtained were subjected to antibacterial testing as above.

Thin layer chromatography (TLC)

TLC of all fractions was carried out using oven baked pre-coated aluminium plates (0.25mm) as the stationary phase and various solvent systems as the mobile phase.Chromatoplates were examined under lights (sunlight and ultraviolet) and iodine crystals in an iodine chamber.

Results

The ethanolic extract (TcE) and its partitioned-soluble portions (TcEc, TcEe, TcEb and TcEr) revealed the presence of several bioactives as shown in Table 1.

Table 1: Preliminary phytochemical screening of crude extracts/ partitioned-soluble portions of *T. cacao*seeds

Bioactive Components	Crude extracts/portions					
	TcP	TcE	TcEc	TcEe	TcEb	TcEr
Carbohydrates	–	++	–	+	+++	++
Reducing sugars	–	++	–	+	+++	++
Combined reducing Sugars	–	++	–	–	++	++
Tannins	–	+++	–	–	++	+
Phlobatannins	–	–	–	–	–	–
Saponins	–	+	–	–	+	+
Flavonoids	–	+++	+	++	+++	++
Steroidal Nucleus	+++	+++	+++	+++	+++	++
Cardiac Glycosides	–	++	–	+	++	++
Free Anthraquinones	–	–	–	–	–	–
Combined Anthraquinones	–	–	–	–	–	–
Alkaloids	+	+++	+	++	+++	+

Key: + = low concentration; ++ = moderate concentration; +++ = high concentration; – = absent.

Antimicrobial activity of the crude ethanolic extract (TcE) and its partitioned-soluble portions (TcEc, TcEe, TcEb and TcEr) in comparison with ampicloxat 4,000µg/ml against test organisms is shown in Table 2.

Table 2: Antimicrobial activity of crude extracts/ partitioned-soluble portions/Ampiclox

Test Organisms	Activity of test compounds against test organisms						
	TcP	TcE	TcEc	TcEe	TcEb	TcEr	Ampiclox
Bacterial strains							
Bacillussubtilis(clinical strain)	–	–	–	–	–	–	+
Staphyloccus spp. (clinical strain A)	–	–	–	–	–	–	+
Staphyloccus spp. (clinical strain B)	–	–	–	–	–	–	+
S. aureus (clinical strain)	–	–	–	–	–	–	+
S. aureus (ATCC 13709)	–	–	–	–	–	–	+
S. epidermidis (clinical strain)	–	–	–	–	–	–	+
Escherichia coli (clinical strain)	–	–	–	–	–	–	–
E. coli (ATCC 9637)	–	+	–	–	–	–	+
Proteus spp. (clinical strain)	–	–	–	–	–	–	+
Pseudomonas aeruginosa (clinical strain)	–	–	–	–	–	–	+
P. aeruginosa(ATCC 27853)	–	–	–	–	–	–	+
Salmonella gallinarium(clinical strain)	–	–	–	–	–	–	+
S. paratyphi C (clinical strain)	–	–	–	–	–	–	+
S. typhi(clinical strain)	–	–	–	–	–	–	+
Fungal strains							
Candida albicans (clinical strain)	–	–	–	–	–	–	+
C. albicans (ATCC 10231)	–	–	–	–	–	–	+

Key: + = activity; – = no activity.

The least concentration of the active ethanolic extract (TcE) that inhibited the growth of*E. coli* is shown in Table 3.

Table 3: Minimum inhibitory concentration (MIC) of TcE

	Concentration of TcE (µg/ml)								
Test Organism	4000	3750	3500	3250	3000	2750	2500	2250	2000
E. coli (ATCC 9637)	+	–	–	–	–	–	–	–	–

Key: + = activity; – = no activity.

The least concentration of the active ethanolic extract (TcE) that yielded no growth against *E. coli* after incubationis shown in Table 4.

Table 4: Minimum bacteriocidal concentration (MBC) of TcE

	Concentration of TcE (µg/ml)								
Test Organism	4000	3750	3500	3250	3000	2750	2500	2250	2000
E. coli (ATCC 9637)	+	–	–	–	–	–	–	–	–

Key: + = activity; – = no activit

Fractionation of active ethanolic extract (TcE) using column chromatography gave rise to four major fractions as shown in Table 5.

Table 5: Fractions collected from crude ethanolic extract of *T. cacao*seeds (TcE)

Fraction number	Code	Eluting solvent	Solvent system for TLC	No of Spots	R_f	Color		
						Sunlight	U.V.	I_2
Jan-42	TcE_1	$CHCl_3$ (100%)	Petroleum ether: $CHCl_3$ (3:1)	6	0.97	Brown	No colour	Brown
					0.88	Bright Yellow	Yellow	Golden brown
					0.72	,,	,,	,,
					0.63	,,	,,	,,
					0.54	No colour	Bright Blue	Brown
					0.41	Brown	No colour	,,
43-87	TcE_2	$CHCl_3$: MeOH (19:1)	$CHCl_3$: MeOH: H_2O (10:3:1)	4	0.84	Faint Yellow	Bright Blue	Brown
					0.76	Faint Yellow	,,	Golden brown
					0.5	Yellow	Faint Yellow	,,
					0.42	Brown	,,	Brown
88-125	TcE_3	$CHCl_3$: MeOH (9:1)	$CHCl_3$: MeOH: H_2O (7:3:1)	3	0.49	Yellow	Yellow	Golden Brown
					0.3	Faint Yellow	,,	,,
					0.21	,,	,,	,,
126-171	TcE_4	$CHCl_3$: MeOH (8:2)	$CHCl_3$: MeOH: H_2O (5:3:1)	3	0.33	Yellow	Yellow	Golden brown

		0.2	,,	,,	,,
		0.1	,,	,,	,,

Antibacterial assay of the four fractions (TcE_1-TcE_4) obtained from fractionation of crude ethanolic extract (TcE) against *E coli* (ATCC 9637) is shown in Table 6.

Table 6: Antibacterial activity of column fractions of TcE

Fractions	Antibacterial activity at 4,000µg/ml.
TcE_1	-
TcE_2	+
TcE_3	+
TcE_4	+

Key: + = activity; – = no activity.

The least concentration of the three active fractions of the active ethanolic extract (TcE) that inhibited the growth of *E. coli* (ATCC 9637) is shown in Table 7.

Table 7: Minimum inhibitory concentration (MIC) of active column fractions of TcE

	Concentration of fractions (µg/ml)								
Fractions	4000	3750	3500	3250	3000	2750	2500	2250	2000
TcE_2	+	+	+	–	–	–	–	–	–
TcE_3	+	+	+	+	–	–	–	–	–
TcE_4	+	+	+	+	–	–	–	–	–

Key: + = activity; – = no activity.

The least concentration of the three active fractions of the active ethanolic extract (TcE) that yielded no growth against *E. coli* (ATCC 9637) after incubation is shown in Table 8.

Table 8: Minimum bacteriocidal concentration (MBC) of active column fractions of (TcE)

	Concentration of fractions (µg/ml)								
Fractions	4000	3750	3500	3250	3000	2750	2500	2250	2000
TcE_2	+	+	+	–	–	–	–	–	–
TcE_3	+	+	+	–	–	–	–	–	–
TcE_4	+	+	+	+	–	–	–	–	–

Key: + = activity; – = no activity.

Discussion

Crude extraction carried out on the seeds of *Theobroma cacao* using a non-polar and polar solvent, revealed thatethanol (TcE) extracted more of the bioactives than petroleum ether (TcP). Successive partitioning of TcE between water and solvents of less polarity showed that the bioactives were extracted more into the polar solvents-butanol (TcEb) and water (TcEr), indicating a significant presence of polar components in the ethanolic extract of *T. cacao*. Generally, polarity of solvents will affect the quantity and types of bio-molecules, eluted from extracts; more polar solvents most often elute more active molecules (Eloff, 1998b). The ethanolic extract (TcE) revealed the strong presence of tannins, flavonoids, steroidal nucleus and alkaloids, while the petroleum ether extract revealed the strong presence of steroidal nucleus

only. The butanol-soluble portion (TcEb) was richer in bioactives, while the chloroform-soluble portion (TcEc) was the least (Table 1).

The result of inhibitory effect of the extracts/portions of seeds of *T. cacao* (Table 2) revealed that only *Escherichia coli* (ATCC 9637) was sensitive to the crude ethanolic extract (TcE) at 4000µg/ml, an organism which is a normal inhabitant of the human and animal intestine and a common cause of diarrhoea (not all strains) and urinary tract infections (Timbury *et al*., 2002).The potency of the crude ethanolic extract against *E. coli* could be as a result of itsbeing rich in various bioactive components (Table 1). Compounds like tannins, saponins, alkaloids and flavonoids have been linked to, or suggested to be involved with antimicrobial activity (Palombo, 2006). Possible synergism between these bioactive components could also account for the observed effect (Okoli&Iroegbu, 2005). The crude petroleum ether extract (TcP) and all the partitioned-soluble portions of TcE did not inhibit the growth of any of the test organisms.Their antimicrobial potency of such extracts/portions could be enhanced at higher concentration. Ampiclox displayed a better efficacy than the crude extracts and soluble portions. Most often, crude plant preparations exhibit lower antimicrobial activity than pure antibiotics (Iroegbu&Nkere, 2005).

Further purification of the active crude ethanolic extract (TcE) using column chromatography (Table 5) gave rise to four major fractions (TcE_1–TcE_4), of which fractions TcE_1 and TcE_2gave rise to more of non-polar components, while fractions TcE_3 and TcE_4 yielded polar components. Fluorescencing spots that appear as various colors, such as red, brown, yellow, green, black, purple etc can be achieved by UV irradiation of substances at 254nm and 366nm (Sherma, 2005). This is indicative of the presence of polycyclic aromatic hydrocarbons and conjugated compounds, such as flavonoids, phenolic acids and some unsaturated compounds (Yrjonen, 2004). These classes of compounds were detected in TcE(Table 1).Absorption of iodine vapor from crystals in a closed chamber produces brown spots with almost all organic compounds except for some saturated alkanes (Kovar&Morlock, 1996).Generally, knowing the number and relative amounts of components in an extract aids in planning further the analytical and separation steps to be employed to the extract to enhance purification and isolation of plant constituents (Fried andSherma, 1999).

Antibacterial assay of the obtained fractions (Table 6) revealed that only fractions TcE_2– TcE_4 displayed inhibitory activity against *E. coli* (ATCC 9637).This is an indication that these active fractions which are expectedly more polar than TcE_1 contains more of mid polar/polar constituents, which probably means the observed better activity of fractions TcE_2–TcE_4 could be due to the presence of these mid polar/polar bioactives. Polar components have been reported to exhibit significant antibacterial/antimicrobial activities against some pathogens (Nazemi*et al.*, 2010).

The MIC values forTcE, TcE_2,TcE_3 and TcE_4 was 4,000µg/ml (Table 3), 3500µg/ml, 3250 µg/ml and 3250 µg/ml (Table 7) respectively, while their MBC values was 4,000µg/ml (Table 4), 3500µg/ml, 3500 µg/ml and 3250 µg/ml (Table 8) respectively.This shows that the active fractions may be more useful in the treatment of diseases caused by *E. coli.* It also indicates that the inhibitory effects produced bythe ethanolic extract and its fractions are only bacteriostatic in action (Babayi*et al*., 2007).

Conclusion

The crude ethanolic extract and its fractions displayed in-vitro inhibitory activities against *E. coli* (laboratory strain) at 4000µg/ml than its partitioned-soluble portions. This suggests that with further fractionation, purification and isolation, the seeds of *T. cacao* couldprovide veritable source(s) of active pure antimicrobial agents.

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