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Full Length Research Paper

Effects of fractionation on antibacterial activity of crude extracts of *Tamarindus indica*

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Column chromatographic fractionation of the crude ethanolic extract of the stem bark of *Tamarindus indica* yielded six fractions (TiA - TiF). Among these, TiB showed about five tracks: TiC, TiD and TiE, two tracks each, on thin layer chromatography (TLC). Fractions TiC, TiD and TiE were re-eluted with different solvent systems and each yielded two sub-fractions, while fraction TiB yielded four. All fractions and sub-fractions tested for antibacterial activity *in vitro* using the agar well diffusion technique. TiA showed activity against 100% of the test gram negative bacterial strains and 60% of the gram positive strains; TiB, TiC, and TiD each showed activity against 71.4% of the gram negative test strains and 100, 80 and 60%, respectively, of the gram positive strains. Fractions TiE and TiF, respectively, showed activity against 42.9 and 14.3% of the gram negatives and 60 and 20% against the gram positives bacteria. The crude extract and Ciprofloxacin (control), respectively, were active against 57.1 and 100% of the gram negatives; and 80 and 100% of the gram positives. The activities of the sub-fractions of TiB, TiC, TiD and TiE against the test strains varied from those of the parent fractions. The phytochemistry of these fractions showed varied contents of tannins, saponins, flavonoids, alkaloids, antroquinone, glycosides and terpene.

Key words: Fractionation, chromatography, plant extract, *Tamarindus indica*, antibacterial activity, phytochemistry.

INTRODUCTION

Plants produce a good deal of secondary metabolites which have benefited mankind in various ways including treatment of diseases (Elaine et al., 2002). These metabolites serve different purposes in the plant, including growth regulation, allelopath, defense against predators and infections or they may be waste products. Outside their intrinsic uses in the plant, these secondary metabolites have variously been shown to exhibit interesting biological and pharmacological activities and are important as prophylactics, chemotherapeutics or have served as the starting points in the development of modern medicines (Verpoorte, 1998). Thus, a crude plant Extract is a complex mixture and its evaluation for the large array of compounds in the complex mixture may interact antagonistically interfering with or masking the activity of one another. Secondly, the vast majority of active compounds in crude extracts is present at a very low concentration and therefore may not show high specific activity.

One approach to solving these problems has been to separate the compounds to greater purity and to concentrate them by various processes, including by chromatography (Jean et al., 2001). It is not always, however, that fractionation of crude extracts improves activity in spectrum and or in specificity and intensity. The efficacious use of crude extracts or concoctions by herbal healers immediately suggests activity of components singly or synergistically in combination. Besides, combinations of these components in nature may interact to reduce toxicity. Hence, purification of crude extracts to concentrate the active principles in line with modern pharmacological practice is thought to result, sometimes, in loss of activity and/or increase in toxicity.

Tamarindus indica L., (Tamarind), family, Leguminosae, is widely used as both food and medicine in many West African communities (Anon, 1986; Morton, 1987). The pulp

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Abbreviations: TLC, Thin layer chromatography; **CFU**, colony forming unit; **IZD**, inhibition zone diameters.

extracts has been documented as antipyretic, antiscorbutic, laxative, carminative and remedy for biliousness and bile disorder (Raimondi, et al., 2003; Morton, 1987; Iwu, 1993). Furthermore, extracts of various parts of the plant have been reported to have antibacterial properties (Doughari, 2006). This study was undertaken, therefore, to fractionate crude ethanolic stem bark extracts of *T. indica* by column chromatography and evaluate the fractions for antibacterial activity as required for inclusion of herbal medicines in orthodox medical practice.

MATERIALS AND METHODS

Plant materials

The stem bark of *T. indica* plant evaluated was obtained from More, Sokoto South Local Government Area, Sokoto State, Nigeria. The plant was taxonomically identified at the Department of Botany, University of Nigeria, Nsukka and a voucher specimen deposited in the herbarium of the Department.

Extractions of the plant materials

Fresh stem bark of *T. indica* were rinsed thoroughly in running tap water, chopped to tiny pieces and air dried in the dark at room temperature (~ 28°C). The dried stem bark was pulverised into powder using a mechanical hammer mill. A 50.0 g weight of the milled material was macerated into 200 ml of absolute ethanol (Sigma-Aldrich) and left to stand for about four (4) hours. The preparation was filtered through Whatman No. 1 paper and filtrate concentrated to dryness in a steady air current. The extract was stored in sterile containers at a temperature of 4°C until further used.

Fractionation of extract

The crude ethanolic extract of the stem bark of *T. indica* was re-constituted in absolute ethanol and spotted on analytical TLC (silica gel G_{600}, 0.25 mm thickness) and the following solvent systems and ratios used as mobile phase to determine the eluent with optimum performance; benzene/ethyl acetate 9:1, 8:2, 7:3, 6:4, and 1:1; ethanol/ethyl acetate 9:1, 8:2, 7:3, 6:4 and 1:1; methanol/ ammonia/ water 8:1:1, chloroform/ethanol 9:1, chloroform/ethanol 1:1 and dichloromethane. After each separation, the TLC plate was exposed to iodine fumes in a chamber. The solvent system giving the best resolution was adopted for column fractionation. Column separation of the extracts was carried out with a glass column of internal diameter 80 mm and length 100 cm (Quickfit, England). Sufficient quantity of a column grade silica gel (120 - 200 mesh size) was wet-packed using benzene/ ethylacetate (6:4) solvent system. A 40 g amount of the crude extract was first dissolved in 20 ml of ethanol, and then mixed with about 20 g of the silica gel to become slurry. The slurry was loaded onto the wet packed column and continuously eluted with the mobile phase (ethanol/ ethylacetate, 6:4). Approximately 20 ml aliquots of eluent were collected while observing the distance traveled by the sample down the column; in addition, bands of the same sample formed on TLC were also monitored. The fractions showing similar TLC mobility and band formation were pooled and the solvent evaporated under a steady air current at room temperature. Fractions which did not give single sharp band on the TLC were re-fractionated using the same silica gel column but different solvent system. The solvent systems employed for this refraction were benzene/ethylacetate (6:4), ethanol/ ethylacetate (6:4), dichloromethane and methanol.

Phytochemical screening

The crude extract, fractions (TiA - TiF) and sub-fractions from re-fractionated fractions were screened for the presence of alkaloids, saponins, tannins, anthraquinones, glycosides, flavonoids, reducing sugar, carbohydrates and sterols using standard phytochemical methods (Trease and Evans, 1978; Harbone, 1998).

Test bacterial strains

Clinical isolates of *Staphylococcus aureus* from a case non-gonococcal urethritis and *Escherichia coli* from a case of gastroenteritis; and two strains of typed *Bacillus cereus* (NRRL 14724 and NRRL 14725) were collected from the Clinical Diagnostic Laboratory, Department of Microbiology, University of Nigeria, Nsukka. Typed strains of *Pseudomonas aeruginosa* (ATCC 10145), *E. coli* (ATCC 11775), *Bacillus subtilis* (ATCC 6051), and *S. aureus* (ATCC 12600) were obtained from Bioresources Development and Conservation Project (BDCP), Nsukka. *Salmonella kintambo* (SSRL 113) was supplied by the Veterinary Microbiology and Pathology Laboratory of the University of Nigeria, Nsukka. Each test bacterial strain was purified by re-isolating severally on Mueller Hinton agar (Oxoid) and emergent discrete colonies picked and identity reaffirmed after characterization by standard bacteriological method (Cheesbrough, 1984). Stock cultures were maintained in nutrient agar slants at +4°C.

Assaying extracts for antibacterial activity

Fractions were assayed for antibacterial activity using the agar well diffusion technique. Inoculum of test bacterial was standardized by McFarland Nephelometry (NCCLS M2-A5, 1993); thereafter, Gram-positive bacteria were adjusted to 1.0 x 10^6 CFU/ml and gram-negative bacteria to 5 x 10^5 CFU/ml (NCCLS M2-A5, 1993). A 100 µl volume of the standardized test bacterial suspension was seeded and spread evenly on to each sterile Muller Hinton agar plate so as to achieve a confluent growth. The plates were allowed to dry and a sterile 6.0 mm-diameter cork borer was used to drill wells in the agar plates. The extracts were reconstituted with sterile distilled water to a concentration of 62.5 mg/ml; and 100 µl introduced in triplicate wells in the MHA cultures. The plates were allowed to stand for 2 h at room temperature for diffusion to take place and then incubated at 37°C for 24 h. The inhibition zone diameter was measured to the nearest mm.

RESULTS

Column chromatography of the crude ethanolic extract of *T. indica* stem bark yielded six fractions designated TiA, TiB, TiC, TiD, TiE and TiF (Table 1). On subsequent subjection of the fractions to TLC, TiA and TiF, each showed a single band whereas TiB, TiC, TiD and TiE showed 5, 3, 2 and 2 bands, respectively. The pH and yield of the stem bark crude ethanolic extracts and fractions of *T. indica* is shown in Table 2. Fraction TiB had more yield (12.0%) than others and TiF, the least (4.5%). The pH values of both crude and fractions ranged from 3.74 (fraction TiA) to 4.61 (crude extract). On re-fractionation, TiB yielded further four distinct fractions

Table 1. Chromatographic separations of the stem bark of *Tamarindus indica*.

Sample	Products of first fractionation	Tracks on TLC	Products of re-fraction	Tracks on TLC
Crude ethanol extract of stem bark	TiA	1	-	-
	TiB	5	B1, B2, B3, and B4	1
	TiC	3	C1 and C2	1
	TiD	2	D1 and D2	1
	TiE	2	E1 and E2	1
	TiF	1	-	-

Table 2. The yield and pH of crude extract of *Tamarindus indica* and its fractions.

Fractions	pH	Yield	% yield
Crude	4.61	4.50	9.0
TiA	3.74	2.3	5.79
TiB	4.21	4.8	12.0
TiC	4.18	3.6	9.0
TiD	4.21	3.9	9.75
TiE	4.43	2.4	6.0
TiF	4.32	1.8	4.50

Table 3. Phytochemical composition of initial fractions of crude extract of the stem bark of *Tamarindus indica*.

Constituents	TiA	TiB	TiC	TiD	TiE	TiF	Crude
Carbohydrates	-	-	+	+ +	+ +	+ +	+ +
Reducing sugar	-	-	-	+	+	+	+
Tannins	+	+ +	-	-	-	-	+
Flavonoids	+ +	+ +	+ + +	+	+	+	+ + +
Anthroquinone	-	+ + +	+	-	-	-	+ +
Saponins	+ +	+ + +	+ + +	+ + +	+ +	-	+ + +
Alkaloids	-	+ + +	+	+	+	-	+ + +
Cyanogenic glycoside	-	+	+ +	-	-	-	+ +
Terpenes	-	-	+	-	-	-	-
Sterol	-	-	-	-	-	+	-

- = Not detectable; + = present in trace quantity; + + = moderately present; + + + = highly present.

(B1, B2, B3 and B4) which expressed single track on TLC each; the others yielded two further fractions each, namely, TiC C1, C2), TiD (D1, D2) and TiE (E1, E2) (Table 1). The phytochemical analysis showed that TiA, TiB, TiD and TiE contained saponins, flavonoids and alkaloids. Tannins were only detected in TiA and TiB; anthroquinone, terpene and cyanogenic glycosides only in TiC and Sterol only in TiF (Table 3). Sub-fractions of TiB1, TiB2, TiB3, TiB4, TiC1 and TiC2 all contained saponin while alkaloids were detected in TiB4, cyanogenic glycolsides in TiB3, Terpene in TiB1 and sterol in TiE1 and TiE2 (Table 4).

All six parent fractions TiA to TiF from the crude extract showed antibacterial activity with IZDs (inhibition zone diameters) ranges of 9.0 ± 1.42 mm against *S. kintambo* SRRL; 113 to 13.0 ± 0.5 mm against *B. cereus* NRRL; 14724 for TiA; 9.50 ± 0.25 mm against *Proteus mirabilis* (clin.) to 17.0 ± 0.71 mm against *P. aeruginosa*; ATCC 10145 for TiB; 9.0 ± 1.2 mm against *S. aureus* ATCC; 12600 to 15.0 ± 0.25 mm against *E. coli* (clin.) for TiC; 9.0 ± 1.2 mm against *S. aureus* (clin.) to 15.0 ± 0.25 mm against *E. coli*; ATCC 11775 for TiD; 10.0 mm against *S.* aureus ATCC 12600 to *B. subtilis*; ATCC 6051 for TiE; and 10.0 ± 0.25 mm against *P. aeruginosa* ATCC; and 10145 to 12.0 ± 0.25mm against *S. aureus* for TiF (Table 5). Sub-fractions TiB1, TiB3, TiB2 and TiB4, respectively,

Table 4. Phytochemical composition of re-fractions.

Phytoconstituent	TiB				TiC		TiD		TiE	
	B1	B2	B3	B4	C1	C2	D1	D2	E1	E2
Carbohydrates	-	-	-	-	+ +	+ + +	+	+	-	+ +
Reducing sugar	-	-	-	-	+	+	+	+	-	+
Tannins	-	+ +	-	+	-	+	-	-	-	-
Flavonoids	-	+ +	-	-	-	+ + +	-	+	-	+
Anthroquinone	-	+	-	+	-	+	-	-	-	-
Saponins	+ +	+	+	+	+ +	+ +	+ +	+ +	-	-
Alkaloids	-	+	+	+ + +	-	+	-	+	-	-
Cyanogenic glycoside	-	+	+ + +	-	-	+	-	-	-	-
Terpenes	+ + +	-	-	+	+ +	+	-	-	-	-
Sterol	-	-	-	-	-	-	-	-	+	+

- = Not detectable; + = present in trace quantity; + + = moderately present; + + + = highly present.

Table 5. The antibacterial activities of the fractions and crude ethanolic extract of *Tamarindus indica* stem bark.

Bacterial strain	Mean inhibition zone diameter (125 mg/ml)							
	Crude extract (sbf)	Ti (A)	Ti (B)	Ti (C)	Ti (D)	Ti (E)	Ti (F)	Ciproflox (20 µg/ml)
E. coli	20.50 ± 0.71	11 ± 0.25	16 ± 0.71	16 ± 0.71	15 ± 1.2	13 ± 0.25	0	26.25 ± 0.25
E. coli ATCC 11775	8.50 ± 0.71	12 ± 0.71	15.5 ± 0.71	15.5 ± 0.71	15 ± 0.25	0	0	32.85 ± 0.25
Salmonella typhi	0	11 ± 0.5	0	0	0	0	0	24.0 ± 0.25
Salmonella kintambo SSRL 113	0	9 ± 1.42	0	11 ± 1.0	12 ± 0.25	10 ± 0.25	0	21.80 ± 0.60
Staphilococcus aureus	14.50 ± 0.71	0	16.50 ± 0.25	9 ± 0.71	9 ± 1.2	14 ± 1.20	12 ± 0.25	25.85 ± 0.25
Staph. aureus ATCC 12600	0	10.50 ± 0.50	12 ± 0.71	9 ± 1.2	0	10 ± 0.60	0	22.25 ± 0.45
Pseudomonas aeruginosa	21.0 ± 1.24	10 ± 0.25	13.50 ± 0.71	8	8.50 ± 0.25	0	0	25.85 ± 0.25
Pseudomonas aeruginosa ATCC 10145	17.0 ± 0.0	12.50 ± 0.5	17 ± 0.71	14 ± 1.2	14 ± 0.25	12 ± 0.60	10 ± 0.25	23.25 ± 0.25
B. subtilis ATCC 6051	12.50 ± 0.71	11 ± 1.42	12 ± 0.50	14.50 ± 1.0	0	14 ± 0.25	0	31.0 ± 0.75
Proteus mirabilis	15.0 ± 0.0	10 ± 0.25	9.50 ± 0.25	0	0	0	0	24.60 ± 1.0
B. cereus NRRL 14724	15.0 ± 0.0	13 ± 0.5	16.50 ± 2.1	0	11 ± 0.25	0	0	27.25 ± 0.25
B. cereus NRRL 14725	10.0 ± 1.41	0	13 ± 0.71	11 ± 0.65	10 ± 0.25	0	0	26.0 ± 1.00

Sbf = Stem bark fraction; ciproflox = ciprofloxacin antibiotic.

showed activity against 57.0, 42.9, 28.6 and 14.2% of the test gram negative test bacterial strains compared with the 71.43% spectrum of activity of the parent fraction (Figure 1). Similarly, TiB1and TiB2 were active against 20% and TiB3 and TiB4 against 60 and 40%, respectively, of the gram positive test bacterial strains (Figure 2). Sub-fraction TiC1 was active against all the gram negative strains and 60% of the gram positive strains; TiC2 showed activity against 42.9% of the gram negative strains and 40% of the gram positive strains. Also, TiD2 showed activity against 71.40% of the gram negative bacterial strains and 60% of the gram positives; TiE1 showed activity against 57.10% of the gram negatives and 40% of the gram positives but TiE2 showed activity against 41.86% of the gram negatives and 60% of the gram positives, respectively.

DISCUSSION

All the fractions and sub-fractions had acid pH (3.74 - 4.61) which means that the aggregate hydrogen ion (H^+) effect of the component compounds is acidic and consistent with the reported pH of extracts from most

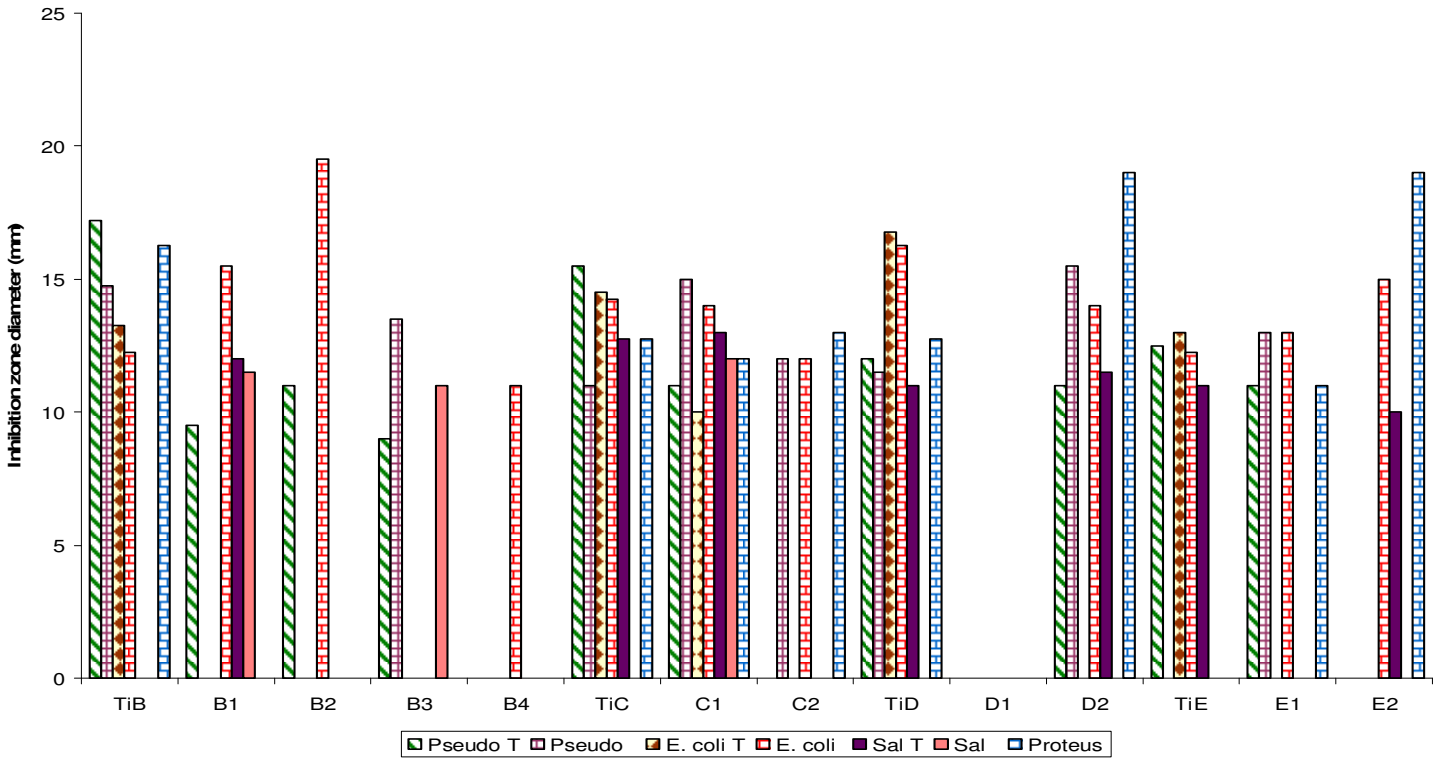

Figure 1. Comparative susceptibility pattern of gram negative test bacterial strains to chromatographic fractions and re-fraction.

plant materials (Anderson, et al., 2001; Dnyaneshwar et al., 2003). The phytochemicals detected in the crude and fractions such as flavonoids, tannins, glycosides and saponins have been associated with antimicrobial activity by other workers (Marjorie, 1999; Mahajan and Badgujar, 2008). However, since there is a family of these compounds, there may be a need to determine which specific compound(s) amongst them exhibits the antimicrobial activity. Among the six fractions got initially from the parent crude ethanolic extract of the stem bark, TiF showed the least antibacterial activity. With those that showed better antibacterial activity, the range and type of organisms showing susceptibility varied with fraction, which indicates that there were several types of compounds with antibacterial activity among the phyto-chemical constituents of the stem bark of the *T. indica* plant. Thus, TiB probably contained the highest proportions of these antibacterial compounds followed by TiC, TiA, TiD and TiE, in that order. It is not surprising, therefore, that TiB showed a wider spectrum of activity than other fractions; but whether this superiority of activity means that it contains more potent antimicrobial principles or it contains compounds acting together synergistically or additively needs to be ascertained. On the other hand, the superior activity could be due to the presence of higher concentrations of the bioactive components because of the dose response curve, which showed that the higher the concentration of extract, the greater the inhibition zone diameter (U. U. Nwodo and C. U. Iroegbu, unpublished observation). The same caution is exercised in the interpretation of the observed greater proportion of the organisms susceptible to fraction TiA.

Fractionation and re-fractionation in some cases resulted in improved activity but in others resulted in loss of activity. For example, when TiB was re-fractionated, one of its sub-fractions, TiB4, showed no activity against type *P. aeruginosa* strains and TiB3 showed no activity against clinical strain of *P. aeruginosa* unlike the parent fraction TiB. Similarly TiB1, TiB2, TiB3 and TiB4 lost activity against *E. coli* ATCC 11775; only TiB3 had activity against *E. coli* (clin.) in contrast to the parent TiB. These represent a situation where purification leads to loss of activity suggesting that components of TiB may have acted synergistically or additively to produce the activity observed with the parent fraction. However, TiB3's activity against *E. coli* (clin.) indicates that it is probably a singly active constituent. Conversely, TiC1 exhibited activity against *Salmonella* sp. in contrast to the parent fraction, TiC and sister sub-fraction TiC2 which showed no activity against this strain. It is yet to be ascertained that this is a case of TiC2 antagonizing TiC1 in the parent TIC, thus hindering its activity against the strain. All these show that purification of crude extracts could produce loss or gain of activity depending on the nature of interaction (antagonism or synergism/additivity) between the constituent compounds of the extract.

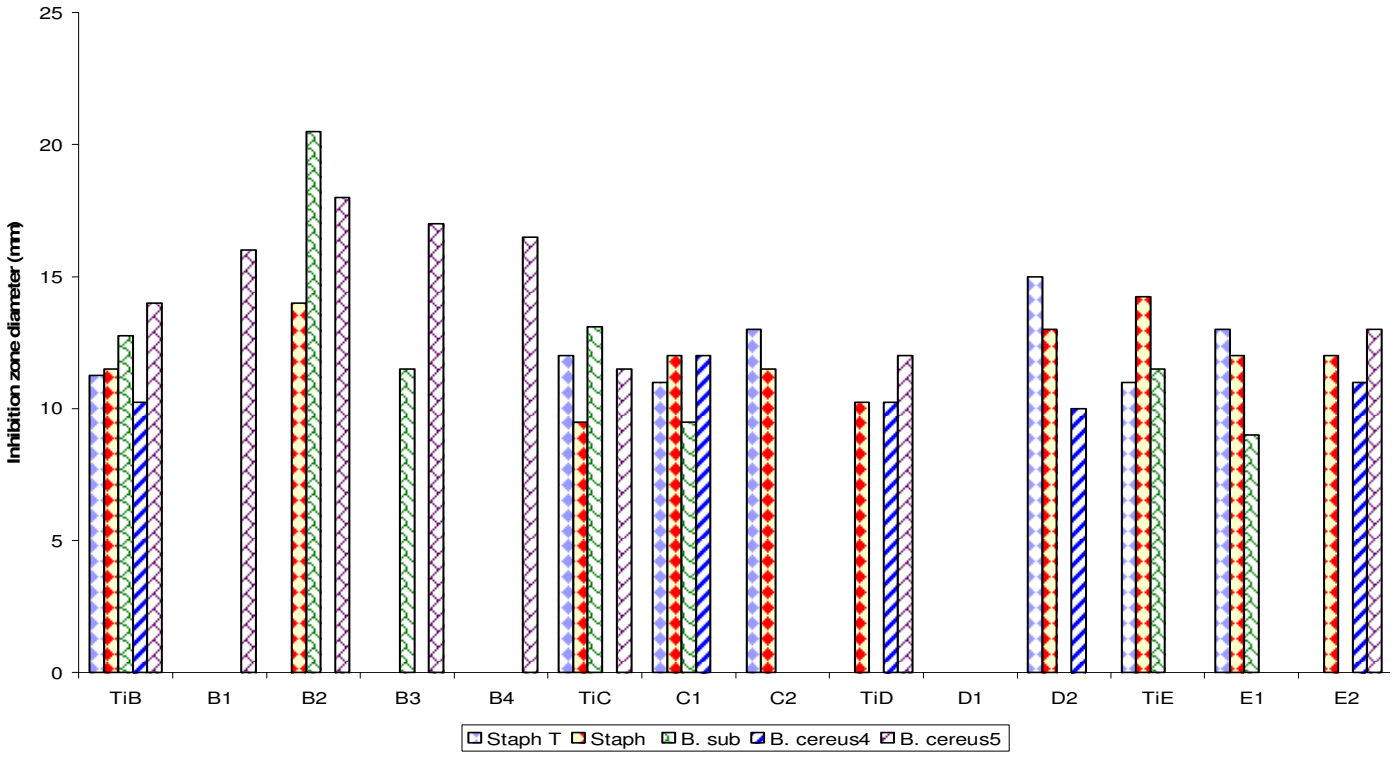

Figure 2. Comparative susceptibility pattern of Gram positive test bacterial strains to chromatographic fractions and re-fraction.

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