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Hand print from 8.5 year old boy after playing outside. photo by Tasha Sturm

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Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta***.** 50th Annual Symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. www.molecularsystemsbiology.com. DOI:10.1038/msb.2008.24

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Effect of *Lactobacillus acidophilus* and *Lactobacillus plantarum* on the quality of yogurt

HIND ALI MOHAMED SIDDIQ, OMER IBRAHIM AHMED HAMID♥
College of Graduate Studies, Sudan University of Science and Technology. Khartoum, Sudan. ♥email: omerhi30@sustech.edu, omerhi30@yahoo.com

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Abstract. *Siddiq HAM, Hamid OIA. 2017. Effect of* Lactobacillus acidophilus *and* Lactobacillus plantarum *on the quality of yogurt. Bioteknologi 14*: *25-31.* The aim of this study was to evaluate the effect of adding different levels of culture starter and storage period to conventional yogurt culture. Eight liters of fresh cow milk were purchased from a dairy farm located at the College of Animal Production Science and Technology, Khartoum Sudan. The milk was pasteurized at 90°C for 30 minutes then cooled to 45°C. The pasteurized milk was then divided into four equal portions. Four treatments were carried out. The first treatment was the control sample, namely yogurt which was from conventional yogurt culture starter. In the second, third and fourth treatments, respectively, 50%, 75% and 100% of culture starter adjunct (*Lactobacillus acidophilus* and *Lactobacillus plantarum*) were added. The inoculated milk was incubated in all treatments at 43 °C for three hours. After complete coagulation, the yogurt samples were cooled by refrigeration with a temperature of 4°C. Chemical composition and sensory evaluation were carried out on the yogurt samples in all treatments at intervals of 1, 5 and 10 days. The results indicated that significant variations ($P<0.05$) were found in the fat %, acidity % and protein % while total solids and the ash % showed no significant difference ($P>0.05$) due to the culture starter adjunct addition. However, the storage period had significant difference ($P<0.05$) on the fat, protein, total solids, and acidity while the ash content was not affected by storage period. The sensory characteristics of the yogurt samples were found to be not affected significantly ($P>0.05$) by the storage period, except the texture and overall acceptability. The addition of starter cultures had significant variations on the color, flavor, texture, taste, and overall acceptability. The results revealed that the treatments with 50% and 75% showed higher sensory scores than others.

Keywords: *Lactobacillus acidophilus, Lactobacillus plantarum,* yogurt

INTRODUCTION

Around the world, cow milk is used to produce fermented milk including yogurt. In the Indian subcontinent, buffalo milk and blends of buffalo and cow milk are used widely for Dahi (a type of fermented milk) made by the help of mixed mesophilic cultures (Aneja et al. 2002). Buffalo milk is the base for making yogurt using thermophilic cultures in several Asian countries, whereas the milk of sheep, goats, and camels are used for fermentation in several Middle Eastern countries. In modern times, yogurt is a significant dairy product worldwide. It is a semi-solid fermented product made from heat-treated standardized milk, mixed by the activity of a symbiotic blend of *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* (Clark and Plotka 2004; Ozer 2010).

In certain countries, the nomenclature "yogurt" is restricted to the product made exclusively from the two lactic cultures. Whereas in other countries, it is possible to label a product as "yogurt" if it is made with yogurt cultures and adjunct probiotic cultures. The more common cultures adjunct is *Lactobacillus acidophilus*, *Bifidobacterium*, *Lactobacillus gasseri* and *Lactobacillus rhamnosus* (Maity and Misra 2009; Chandan and Nauth 2012). Yogurt represents the most popular fermented milk product worldwide and originates from countries around the Balkan and the Eastern Mediterranean Sea (Staff 1998; Walstra et al. 1999). Yogurt also has medical uses because of the probiotic characteristics, in helping on a variety of gastrointestinal conditions and in preventing antibiotic-associated diarrhea (Lourens-Hattingh and Viljoen 2001; Mazahreh and Ershidat 2009).

The art of making Zabadi (yogurt) came to Sudan from Egypt, most likely during the time of the Anglo-Egyptian rule (1898-1956). It was prepared by households, by the cow milk being boiled, cooled, and inoculated by back-shopping from a previous lot. It was then incubated in a warm carrier, where it sours and is then refrigerated. It was consumed with sugar as a dessert or eaten with wheat bread. Sometimes it is fed to babies and is often turned into sauce for porridge (Chandan 1999).

The objective of the study is to determine: (i) The effect of starter cultures (*Lactobacillus acidophilus* and *Lactobacillus plantarum*) on the organoleptic properties of yogurt. (ii) The effect of storage period on the chemical and sensory evaluation of yogurt.

MATERIALS AND METHODS

This study was conducted during the period of October-November 2014 at the Department of Dairy Sciences and Technology, College of Animal Production Science and Technology, Sudan University of Science and Technology, Khartoum Sudan.

Materials

Eight liters of raw cow milk were purchased from the Dairy Farm, College of Animal Production Science and Technology, Sudan University of Science and Technology, Hillat Kuku. The sterilized containers which were used for the collection of samples, culture starter of yogurt (*S. thermophillus and L. bulgaricus*) and culture starter adjunct of *L. acidophilus* and *L. plantarum* (1: 1) were brought from Vitane Pharma Gmbh, 82515 Wolfratshausen, Germany.

Adjunct starter culture preparation

One liter of skim milk was sterilized at 85 ºC for 30 min and cooled to 40-45 °C then inoculated with the culture starter adjunct at the rate of 2% and incubated at 45ºC until coagulation occurred.

The starter culture:

Conventional yogurt and culture starter adjunct were added at the rate of 2% of the milk used for yogurt making.

Yogurt making process

Yogurt was prepared as described by Staff (1998), namely, eight liters of cow raw milk was heated in a water bath at 85 °C for one hour and cooled to 45°C. Then the milk was divided into four portions, the first portion was used as control only with yogurt culture starter. To the 2nd, 3rd ,and 4th portions, 50%, 75% and 100% of culture starter adjunct (*L.acidophillus and L.plantarum*) was added to each respectively and packed into plastic cups (200 mg capacity) in triplicates for each treatment and then incubated at 43°C for 3 hrs. Samples from different treatments were stored in a refrigerator at 4 °C for 1, 5 and 10 days; chemical and sensory evaluation of the yogurt samples were analyzed for the determined period.

Chemical analysis

Fat content

The fat content was determined by Gerber method according to Bradly et al. (1992). In a clean dry Gerber tube, 10 ml of sulphuric acid (density 1.8 g/ml at 20 °C) was poured. 10.94 ml of milk sample was added with amyl alcohol (1-2 ml) to tube. This was followed by the pouring of distilled water into it. The content was thoroughly mixed until no white particles could be seen. The Gerber tube was centrifuged at 1100 revolution per minute (rpm) for 4-5 min. The fat column was then read immediately.

Protein contents

The protein content was determined by Kjeldahl method according to AOAC (1990). (1) *Digestion***:** ten ml of milk were weighed and poured in the flask. Concentrated sulphuric acid (25ml) was added to the flask. The flask was heated until a clear solution was obtained. Then, the flask was removed and allowed to cool. (2) *Distillation***:** the digested sample was poured in volumetric flask (100 ml) and diluted to 100ml with distilled water. Five milliliters of it was distilled using 10 ml of 40% NaOH. The distillate was poured into a conical flask (100ml) containing 25 ml of 2% boric acid plus 3 drops of indicator (bromocresol green + phenolphthalein red). The distillation was continued until the volume in the flask was 75 ml, then the flask was removed from the distillatory. (3) *Titration*: the distillate was titrated with 0.1N HCl until the end point (red color) was obtained. The protein content was calculated by the following equation:

$$Nitrogen\ \% = \frac{T \times 0.1 \times 20 \times 0.014 \times 100}{w}$$

Protein % = N% × 6.38

Where:
T= Titration figure
W = Weight of the original sample
0.1 N = Normality of HCL
0.014 = the atomic weight of nitrogen/100
20 = Dilution factor

Total solid (T.S.) content

The total solid content was determined according to the modified method of AOAC (1990). Three grams of sample was weighed into a dry oven flat-bottomed aluminum dish, and heated on steam bath for 10-15 min. The dish was placed in an oven at 105º C overnight, and then cooled in desiccators and weighed quickly. The weighing was repeated until the difference between the two readings was < 0.1mg. The total solid content was calculated by the following equation:

T.S% = W1/Wo × 100

Where:
W1 = Weight of sample after drying
Wo = Weight of sample before drying

Ash content

The ash content was determined according to AOAC (1990). Five grams of the sample was weighed and put into a suitable crucible and evaporated to dryness on steam bath. Then it was placed in a muffle furnace with temperature of 55-60º C until ashes were carbon-free (2-3 hrs.), then the crucibles were coded in a desiccator, and the weight of the ash content was calculated with the following equation:

Ash% = W1/W× 100

Where:
W1 = Weight of ash
W= weight of sample

Sensory evaluation

Sensory profiling of the yogurt sample was conducted using conventional profiling by untrained panelists according to Larmond (1977). Ten panelists were selected among staff and students at the College of Animal Production Science and Technology, Sudan University of

Science and Technology, Khartoum, Sudan. The panelists were given a hedonic questionnaire (Appendix NO1) to evaluate taste texture, color, and flavor and overall acceptability of coded samples of cow milk yogurt which were stored for different period (1, 5, 10 days). They were scored on a scale of 1-7 (1=not acceptable, 7= acceptable). Each attribute was evaluated in triplicate, and the values were then averaged.

Statistical analysis

Statistical analysis was carried out with SPSS (2008) version 17. General linear model was used for data analysis (Factorial design) to test the effect of culture starter adjunct addition and storage period on the quality of yogurt. Least significant difference (LSD) was used for mean separation between the treatments. Alpha level 5% was used in this study.

RESULTS AND DISCUSSION

Effect of different levels of adjunct starter culture on the chemical characteristics of yogurt

Data in Table 1 showed the effect of culture starter adjunct on the physicochemical composition of set yogurt. The results indicated that the culture starter adjunct had significant ($P< 0.01$) effect on the fat content of set yogurt (Table 1).The highest fat content (3.56± 0. 06%) was for the control yogurt, while the lowest value (3.28±0.06%) was for the one with 75% culture starter adjunct. The titratable acidity of the yogurt samples showed significant ($P<0.05$) variations due to the different level of culture starter adjunct (Table 1). As the level of culture starter adjunct increased, the titratable acidity decreased. However, the lowest acidity (0.94±0.02%) was for the yogurt with 75% culture starter adjunct.

Statistical analysis revealed that culture starter adjunct had no significant ($P>0.05$) effect on the total solids and ash contents of the set yogurt (Table 1). The results of the study demonstrated that (Table 1) addition of the culture starter adjunct had significant effect on the crude protein content. The highest protein content (5.52±0.05%) was for the yogurt with 50% each conventional and culture starter adjunct, whereas the lower one was for the yogurt with 75% adjunct starter culture.

Effect of storage period on the chemical composition of set yogurt:

Data in Table 2 shows the effect of storage period on the chemical composition of the set yogurt. The results indicated that the storage period had significant ($p<0.05$) effect on the fat content of set yogurt (Table2). The highest fat content (3.62 ±0.05%) was on day 1. The lowest value (3.28 ± 0.05%) was at day 5. The titratable acidity of the yogurt samples showed significant ($p<0.05$) variations due to the storage period (Table 2). As the storage period progressed, the titratable acidity increased. However, the highest acidity (1.21±0.02%) was at day 10.

The results of the study demonstrated that (Table 2) the storage period had significant effect on total solid. The highest total solid (12. 52 ± 0.04%) was at day 5. The lowest value (12.30 ± 0.04%) was at day 10. Statistical analysis revealed that storage period had no significant ($p>0.05$) effect on ash content of the set yogurt (Table 2). The results indicated that the storage period had significant ($p<0.05$) effect on the crude protein content. The highest protein content (5.97± 0.05%) was on day 1. The lowest value (4.96 ± 0.05%) was at day 5.

Effect of storage period and levels of culture starter adjunct on chemical composition of yogurt

The results indicated that the storage period and different level of culture starter adjunct had significant ($p<0.05$) effect on the fat content of set yogurt (Table 3).The highest fat content (3.9± 0.15%) was for the control yogurt on day 1. The lowest value (2.8± 0.15%) was for the one with 75% culture starter adjunct on day 5. The results of the study showed that (Table 4) the addition of the culture starter adjunct and the storage period had significant ($p<0.05$) effect on the titratable acidity of the yogurt samples. The highest titratable acidity (1.4±0.03%) was for the one with 50% culture starter adjunct on day 10. The lowest value (0.56± 0.05%) was for the one with 75% culture starter adjunct at day 1.

The results revealed that the storage period and different level of culture starter adjunct had significant ($p<0.05$) effect on the total solid of yogurt samples (Table 5). The highest total solids content (12.9± 0.05%) was for the one with 100% culture starter adjunct at day5, the lowest value (12.2± 0.20%) was for the one with 50% culture starter adjunct at day 10. The storage period and different level of culture starter adjunct had significant ($p<0.05$) effect on the ash of yogurt (Table 6). The highest ash content (0.81± 0.02%) was for the one with 75% culture starter adjunct on day 5 , the lowest value (0.73± 0.15%) was for the control at day 5.

The storage period and different level of culture starter adjunct had significant ($p<0.05$) effect on the crude protein content of set yogurt (Table 7). The highest protein content (6.0± 0.08%) was for the one with 50% culture starter adjunct on 10 days, the lowest value (4.0± 0.04%) was for the one with 75% culture starter adjunct at day 5.

Effect of culture starter adjunct on the sensory characteristics of yogurt

The addition of culture starter adjunct had significant ($p<0.01$) effect on the color of yogurt (Table 8), the highest value (8.07± 0.34) was for the control yogurt while the lowest one (6.80± 0.34) was for the one with 100% culture starter adjunct. The results showed that culture starter adjunct had significant ($p<0.05$) effect on the flavor of yogurt (Table 8).The highest scores (7.20±0.28) was for the one with 75% culture starter adjunct. However, the lowest value (5.60± 0.28) was for the one with 100% culture starter adjunct. The texture and taste of the yogurt samples were found to be affected significantly ($p<0.05$) by the culture starter adjunct addition. The highest texture value (7.33±0.32) was for the one with 50% culture starter adjunct, and the lowest value (5.07±0.22%) was for the one with 100% culture starter adjunct. The highest taste scores

(6.87± 0.41) was for the control yogurt and the one with 50% culture starter adjunct, the lowest value (4.87± 0.41) was for the yogurt with 100% culture starter adjunct.

Overall acceptability of yogurt samples (Table 8) was significantly ($p<0.05$) affected by the culture starter adjunct addition, the highest score (7.53± 0.25) was the one with control yogurt. The lowest value (5.93± 0.25) was for the one with 100% culture starter adjunct.

Effect of storage period on the sensory characteristics of yogurt

The data in Table 9 showed that no significant ($P<0.05$) variations were observed in the color, flavor, and taste of the yogurt samples. However, significant differences ($P<0.05$) were in the texture and overall acceptability of the yogurt samples. The color of the yogurt samples did not change during the storage period, the highest color scores (7.95±1.43) were at day 5 and the lowest value was at day 1.

Table 1. Effect of different level of starter culture on chemical characteristics of set yogurt.

	Chemical composition (%)				
Treatments	**Fat**	**Acidity**	**T.S**	**Ash**	**Protein**
A	3.56± 0. 06a	1.20±0.02b	12. 38±0.05	0. 75±0.02	5. 33± 0.5b
B	3.51±0.06b	1.23±0.02a	12. 40± 0.05	0.80±0.02	5.52±0.05a
C	3.28±0.06d	0.94±0.02d	12.35± 0.05	0. 80±0.02	4.48±0.05 d
D	3.43±0.06c	0.99±0.02c	12.51± 0.05	0. 80±0.02	5. 03±0.06c
Sig	*	**	NS	NS	**

Note: Means with different superscript in the same column are significantly ($p<0.05$) different. A = control yogurt with conventional culture starter, B = yogurt with 50% conventional culture starter and 50% culture starter adjunct, C = yogurt with 75% culture starter adjunct and 25% conventional culture starter, D = yogurt with 100% culture starter adjunct.

Table 2. Effect of the storage period on the chemical characteristics of set yogurt.

Storage/days	Chemical composition (%)				
	Fat	**Acidity**	**T.S**	**Ash**	**C.P**
Day 1	3.62± 0. 05a	0.97± 0.02b	12. 40± 0.04b	0. 79± 0. 02	5. 97± 0.05a
Day 5	3.28±0.05c	1.09±0.02c	12. 52± 0.04a	0. 79±0.02	4.84±0.05c
Day 10	3.43±0.05b	1.21±0.02a	12.30± 0.04b	0. 79±0.02	4.96±0.05 b
Sig	**	**	**	NS	**

Note: Means with different superscript in the same column are significantly ($p<0.05$) different.

Table 3. Effect of the storage period and different level of culture starter adjunct on the fat content (%) of yogurt.

Treatment	Storage period			
	day1	**Day5**	**Day 10**	**Sig**
A	3.9± 0.15	3.5± 0.10	3.3± 0.00	
B	3.7±0.17	3.3±0.06	3.6±0.30	
C	3.3±0.26	2.8±0.15	3.7±0.30	
D	3.6±0.00	3.5±0.11	3.1±0.7	**
Sig	**			

Note: A = control yogurt with conventional culture starter, B = yogurt with 50% conventional culture starter and 50% culture starter adjunct, C = yogurt with 75% culture starter adjunct and 25% conventional culture starter, D = yogurt with 100% culture starter adjunct.

Table 4. Effect of storage period and different levels of culture starter adjunct on the acidity (%) of yogurt.

Treatment	Storage period			
	Day 1	**Day 5**	**Day 10**	**Sig**
A	1.1± 0.05	1.2± 0.05	1.3± 0.01	
B	1.1±0.00	1.1±0.01	1. 4±0.03	
C	0.56±0.05	1.1±0.02	1.1±0.18	
D	1.1±0.04	0.86±0.01	1.0±0.01	**
Sig	**			

Note: A = control yogurt with conventional culture starter, B = yogurt with 50% conventional culture starter and 50% culture starter adjunct, C = yogurt with 75% culture starter adjunct and 25% conventional culture starter, D = yogurt with 100% culture starter adjunct.

Table 5. Effect of storage period and different level of culture starter adjunct on total solid of yogurt.

Treatments	**Storage period**			
	day 1	**day 5**	**day 10**	**Sig**
A	12.4± 0.05	12.4± 0.05	12.3± 0.21	
B	12.5±0.25	12.6±0.15	12.2±0.20	
C	12.5±0.08	12.3±0.05	12.4±0.05	
D	12.4±0.15	12.9±0.05	12.3±0.03	**
Sig	**			

Note: A = control yogurt with conventional culture starter, B = yogurt with 50% conventional culture starter and 50% culture starter adjunct, C = yogurt with 75% culture starter adjunct and 25% conventional culture starter, D = yogurt with 100% culture starter adjunct.

Table 6. Effect of storage period and different level of culture starter adjunct on ash content (%) of set yogurt.

Treatment	**Storage period**			
	Day 1	**Day 5**	**Day 10**	**Sig**
A	0.75± 0.05	0.73± 0.15	0.77± 0.05	
B	0.80±0.00	0.80±0.00	0.80±0.00	
C	0.80±0.00	0.81±0.02	0.80±0.00	
D	0.80±0.00	0.80±0.02	0.80±0.00	**
Sig	**			

Note: A = control yogurt with conventional culture starter, B = yogurt with 50% conventional culture starter and 50% culture starter adjunct, C = yogurt with 75% culture starter adjunct and 25% conventional culture starter, D = yogurt with 100% culture starter adjunct.

Table 7. Effect of the storage period and different level of culture starter adjunct on protein contents of set yogurt.

Treatments	**Storage period**			
	Day 1	**Day 5**	**Day 10**	**Sig**
A	5.6± 0.07	4.9± 0.49	5.5± 0.04	
B	5.1±0.07	5.4±0.07	6. 0±0.08	**
C	5.2±0.07	4.0±0.04	4.2±0.07	
D	5.9±0.07	5. 0±0.07	4.1±0.05	
Sig		**		

Note: A = control yogurt with conventional culture starter, B = yogurt with 50% conventional culture starter and 50% culture starter adjunct, C = yogurt with 75% culture starter adjunct and 25% conventional culture starter, D = yogurt with 100% culture starter adjunct.

Table 8. Effect of the different level of culture starter adjunct on sensory evaluation of yogurt.

Treatment	**Sensory attributes**				
	Color	**Flavor**	**Texture**	**Taste**	**Overall**
A	8.07± 0. 34a	7.00± 0.28 a	7. 6± 0.32a	6. 87± 0.41a	7. 53± 0.25a
B	8.00±0.34 a	7.07±0.28 a	7. 33±0.32a	6. 87±0.41a	7. 47±0.25a
C	7.53±0.34 a	7.20±0.28 a	6.27±0.32b	6.40±0.41 a	7.4±0.25 a
D	6.80±0.34 b	5. 60±0.28 b	5.07±0.22	4.87±0.41b	5.93±0.25b
Sig	*	**	**	**	**

Note: Means with different superscript in the same Column are significantly ($p<0.05$) different. A = control yogurt with conventional culture, B= yogurt with 50% conventional starter and 50% culture starter adjunct C=yogurt with 75% culture starter adjunct and 25% conventional starter D=yogurt with 100% culture starter adjunct.

Table 9. Effect of storage period on the sensory characteristics of set yogurt.

Storage period/day	**Sensory attributes**				
	Color	**Flavor**	**Texture**	**Taste**	**Overall**
Day 1	7.95±1.43	6.35±1.45	6.20±1.91 b	6.30±2.19	6.90±1.4 2 b
Day 5	7.30±2.10	6.75±1.98	6.10±2.02 cb	5.75 ±2.42	6.75±1.58 cb
Day 10	7.55±2.02	7.05 ±1.39	7.40 ±1.93 a	6.70±2.42	7.60 ±1.58 a
Sig	NS	NS	*	NS	*

Note: Means with different superscript in the same column are significantly ($p<0.05$) different.

The flavor of the yogurt samples improved slightly during the storage period. As the storage period progressed, the flavor scores increased, and the highest scores (7.05 ±1.39) were at day 10. Storage period affected the texture of the yogurt samples significantly (P<0.05). The texture scores of the yogurt samples increased with the advancement in storage. Therefore, the highest texture scores (7.40 ±1.93) were recorded on day 10.

Slight improvement in the taste of the yogurt samples were noticed with the longer storage periods. At day 10, the taste scored 6.70±2.42, which was the maximum. The results indicated that the storage period affected the overall acceptability significantly (P<0.05). The lowest overall acceptability scored 6.75±1.58 which were on day 5 while the highest ones (7.60 ±1.58) were at day 10.

Discussion

The addition of culture starter adjunct to the yogurt improved its quality. The results showed (Table 1) that the chemical composition of the yogurt samples significantly (P<0.05) affected by the addition of culture starter adjunct (*L. acidophilus and L. plantarum*). The decrease in the fat content of the yogurt samples with culture starter adjunct could be due to their lipolytic activities. These findings are in accordance with the results of Mutlu and Guler (2005) who observed that the fat content of bio-yogurt ranged from 3.1 to 4.5% during storage.

The titratable acidity of the yogurt samples with culture starter adjunct showed higher values. This might be due to the high potentiality of culture starter bacteria to convert lactose to lactic acid which increases the acidity of yogurt samples, or probably due to its lower buffering capacity and higher content of non-protein nitrogen. These results agree with those reported by Abrahamsen et al. (1991); Salvador and Fiszman (2004).

The protein content of the yogurt samples with 50% culture starter adjunct was higher in comparison to the other treatments (Table 1). The high protein content could be due to preservative effect of culture starter adjunction the protein content. Our findings are in line with the results of Hassan and Amjad (2010) and Janhoj et al. (2006), who showed that the protein contents of low-fat stirred yogurt ranged from 3.4-5.6%.

The total solid and the ash content of the yogurt samples were not affected by the culture starter adjunct addition (Table 1).This result is not in accordance with those of Hassan and Amjad (2010), who reported the total solid of yogurt with different level of starter increased up to 15.60%±0.56%. The insignificant increase in ash contents was because of the loss of CO2 and water during the mixing of yogurt samples. Moreover, the ash content of yogurt samples in this study was lower than those of Akin and Guler (2005) who reported the ash value of probiotic yogurt as 0.95%. This could be due to the action of *L. plantarum*.

The storage period affected the chemical composition of the yogurt significantly (P>0.05) except the ash content (Table 2). The fat content was highest at day 1 then it decreased at day 5. This could be due to the lipolytic activities of the culture starter adjunct. These results were in accordance with those of Salji et al. (1984). The results (Table 2) showed that acidity tends to increase within the 10 days-storage periods. The acidity of the yogurt samples increased significantly as the storage period progressed. This could be due to the breakdown of lactose into lactic acid by the culture starter adjunct; this may also be due to the lower buffering capacity of *Lactobacillus acidophilus* and higher content of non-protein nitrogen and vitamins which are needed for fast growing microorganisms. These findings are in line with those reported by of Nighswonger et al. *19*96 and Salvador and Fiszman 2004.

The total solid of the yogurt samples increased till day 5 (Table 2), then decreased at day 10. This is probably due to the loss of moisture and the proteolytic activities of the culture starter adjunct during storage. These results were in accordance with the findings of Hassan and Amjad (2010) who reported that total solid increased up to 15.60%±0.56 and with those of Abubakar et al. (2005).

The ash content was not affected significantly by storage period (Table 2). These results are not in agreement with those of Akin and Guler (2005), who reported that the ash value is 0.95%. The storage period affected the protein content (Table 2) of the yogurt samples significantly (P<0.05). The decrease in protein could be due to the proteolytic action of the cultures. Similar findings were reported by Janhoj et al. (2006) who showed that the protein contents of low fat stirred yogurt ranged from 3.4-5.6%.

The interaction between the storage period and the addition of culture starter adjunct significantly affected the chemical composition of the yogurt samples in all treatments (Tables 3, 4, 5, 6 and 7). The lowest fat was for the yogurt sample with 75 % culture starter adjunct at day 5. However, the highest fat % was for the control yogurt samples. This could be due to lipolytic nature of the culture starter adjunct that tended to decrease the fat in the yogurt samples.

The mean sensory scores of the organoleptic evaluation and acceptability for the different yogurt samples are shown in Table 8. The statistical analysis revealed that there were significant differences (p<0.05) among the yogurt samples in the sensory attributes due to the use of adjunct cultures.

Culture starter adjunct affected the color of the yogurt samples negatively. As the level of the culture starter adjunct increased, the color scores deteriorated. This could be due to the high lipolytic and proteolytic activities of the culture starter adjunct. These results agree with those of Nuser (2001). The flavor, texture and taste followed the same trend.

The best flavor and texture scores were for the control and yogurt with 50% culture starter adjunct, the improvement in flavor was probably because of high lactic acid contents which controlled the growth of desirable organisms producing flavor compound which was as the result of sugar and protein and fat degradation.

The storage period was not found to affect the sensory characteristics of the yogurt samples (Table 9) significantly (P>0.05) except the texture and over all acceptability. Our results were not in line with those of Nuser (2001). The

improved in texture till day 5 was likely because of proteolytic agents on the protein breakdown, and it resulted in changing the structure of protein matrix and the texture to become soft and compact. While the deterioration in texture, thereafter, at day 10 might be attributed to further hydrolysis of protein at later stages which leads to very fine and mealy structure. The color of the yogurt in this study was not affected by the storage period.

Based on the results of the study, the following conclusions were drawn: (i) The quality of yogurt was relatively improved with the addition of *L. acidophilus and L. plantarum.* (ii) The addition of different level of culture starter adjunct improved sensory evaluation (overall acceptability). (iii) Significant variations in the sensory characteristics and chemical composition of the yogurt with probiotics were found. (iv) The storage period has no significant effect on the sensory characteristics of yogurt.

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Selection and characterization of soil microorganisms in hydrocarbon biodegradation on crude oil contaminated media

MUHAMMAD FAUZI[1,♥], HARI ROEM HARIYADI[2], MIEKE ROCHIMI SETIAWATI[1], ASRI PENI WULANDARI[3], PUJAWATI SURYATMANA[1]

[1]Deapartement of Soil Science and Land Resources Management, Faculty of Agriculture, Universitas Padjadjaran. Jl. Raya Jatinangor, Sumedang 45363, West Java, Indonesia. ♥email: muhammadfauzi2791@gmail.com

[2] Research Center for Chemistry, Indonesian Institute of Sciences. Jl. Cisitu Sangkuriang, Komplek LIPI, Bandung 40135, West Java, Indonesia.

[3]Departement of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor, Sumedang 45363, West Java, Indonesia.

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Abstract. *Fauzi M, Hariyadi HR, Setiawati MR, Wulandari AP, Suryatmana P. 2017. Selection and characterization of soil microorganisms in hydrocarbon biodegradation on crude oil contaminated media. Bioteknologi 14: 32-36.* This study aims to find the microorganisms that have the ability to degrade hydrocarbon in crude oil contaminated media. The crude oil was taken from PT, Pertamina RU VI, Balongan, Indramayu, West Java, Indonesia. This study was carried out from September to October 2016 in Laboratory of Soil Microbiology, Universitas Padjadjaran, Sumedang, West Java, Indonesia. The strains were from the same laboratory and as follows: *Azospirillum* sp., *Acinetobacter* sp., *Pseudomonas cepacia*, *Bacillus subtilis*, *Penicillium* sp., and *Aspergillus niger*. The selection of microbes is based on the characteristic of microorganisms on degrading hydrocarbon from crude oil on media containing crude oil (Total Plate Count = TPC), the capability on degrading the hydrocarbon of crude oil, and the capability of dissolving phosphorus microbes. The results showed that *Acinetobacter* sp. and *Azospirillum* sp. as nitrogen fixation bacteria, had higher cell population than *Azospirillum* sp. While *P. cepacia* and *Penicillium* sp., as phosphorus dissolving microbes group had higher cell population than *B. subtilis* and *A. niger*. On the process of biodegradation of hydrocarbon, *P. cepacia* had higher on efficiency of hydrocarbon than *B. subtilis*, *Penicillium* sp. was more efficient than *A. niger*, and *Acinetobacter* sp. was more efficient than *Azospirillum* sp. The characteristic capability of a microbe in dissolving phosphate substance in media was marked with *halo zone* form on Pikovskaya media in Petri dish, where *P. cepacia* had larger halo zone than *B. subtilis* and *Penicillium* sp. had larger halo zone than *A. niger*.

Keywords: Biodegradation, crude oil, hydrocarbon, microorganisms

INTRODUCTION

Oil spills are one of the major causes of soil damage in the environment. Leakage and oil spills happen during exploration activities, production, refinery, transportation, and petroleum storage (Das and Chandran 2011). Oil spill waste has been estimated as approximately 600,000 ton per year (Kvenvolden and Cooper 2003). WALHI (2007 in Ali 2009) reported that the case of contaminated soil was affected by hydrocarbon from big company activities. Contaminated soil by crude oil causes serious environmental problems, which demotes water and soil quality(DEQ 2011). So it is necessary to turn back soil fertility from toxic content frequently.

According to PP No. 18 1999, hydrocarbon substance of crude oil is classified into dangerous and toxic material (B3) in the soil. B3 poses physical and chemical substance potentially poisons living biology in the soil, and spoils food chain (Napoleon and Probowati 2014). Therefore, B3 is not allowed to be directly disposed into the environment before being neutralized in the refinery. The contaminated soil can be restored into healthy soil using environment friendly technology such as bioremediation. Bioremediation is one of the environment friendly technologies in landfarming treatment (Cookson 1996). A land farming treatment is an ex-situ process to remove pollution.

Crude oil contains inorganic and organic compounds with various form of hydrocarbon. Diversity of hydrocarbons that can contaminate the soil need various hydrocarbon-degrading microorganisms. Every single hydrocarbon-degrading microbe poses a different function. Bacterial groups can degrade aliphatic hydrocarbon groups. While fungi groups can degrade aromatic hydrocarbon groups. Therefore, application of microbe consortium can degrade various hydrocarbons in the soil.

Crude oil hydrocarbons have both complex and simple forms in the soil. Composition of complex hydrocarbon can be degraded by giving the various species of microorganisms until the diversity of hydrocarbon optimally degraded. Whereas the only single microorganism applied into contaminated crude oil media limitedly degrades hydrocarbon (Ghazali et al. 2004). As we know, some microbe groups that degrade hydrocarbon substance are: *Azospirillum* sp. (Gałazka and Gałazka 2015), *Acinetobacter* sp. (Sihag et al. 2013), *Pseudomonas cepacia* and *Bacillus subtilis* (Ghazali et al. 2004), *Penicillium* sp. (Dhar et al. 2014), and *Aspergillus niger* (Flayyih and Jawhari 2014). The selection of microorganisms for applied bioremediation of

hydrocarbons must be considered to be easy to be cultured and able to live in contaminated environments (Mrozik and Piotrowska-seget 2010). The way to identify the various microbes for bacteria or fungi is needed to find the microbe species dominantly degrading hydrocarbon compounds.

MATERIALS AND METHODS

Materials

Crude oil used for this research was from PT. Pertamina RU VI, Balongan, Indramayu, West Java, Indonesia (Figure 1).

Procedures

Media for production of biomass

The isolated soil microorganisms were from the Soil Biology Laboratory Collection, Department of Soil Science and Land Resource Management, Faculty of Agriculture, University of Padjadjaran. The media used to process the hydrocarbon degradation test were Potato Dextrose Agar (PDA)-liquid for *A. niger*; and *Penicillium* sp., Pikovskaya-liquid for *P. cepacia* and *B. subtilis*, and Natrium Agar (NA)-liquid for *Acinetobacter* sp. and *Azospirillum* sp. All microorganisms were grown previously on media-tilt for 3-4 days to pure the strain culture that would produce biomass and would be saved in incubator. Next, the six microorganisms produced their biomass in each liquid chosen media. Biomass was produced with 10 mL volume in 100 mL liquid media which were poured into Erlenmeyer glasses with a volume of 250 mL. These were kept for 7 days and centrifuged at 15 rpm. For ensuring the biomass uniformity morphology of microorganism, a gram staining test was carried out. Thus, the test for degradation process test on crude mixed oil media could be performed.

Preparation for degradation test

Before hydrocarbon degradation test was performed, a morphology test of microbe cell with gram staining method on microbe was done. This was to decide the characteristic of cell wall. Next, the test of hydrocarbon degradation process was performed in the following stages: each liquid media was prepared by mixing 1% of crude oil from the volume of media 100 mL then pour into Erlenmeyer glass 250 mL. The crude oil was from PT. Pertamina RU VI, Balongan, Indramayu, West Java, Indonesia. To culture microorganisms optimally, they were grown in media and centrifuging all inoculant at 150 rpm for a month (30 days). Cells of microbes were harvested to decide the research parameter. Then each bacterial group was compared based on the total population of microorganism, the capability to degrade the hydrocarbon and the capability of microbe in dissolving phosphorus substance (*P. cepacia*, *B. subtilis*, *Penicilium* sp., and *A. niger*). Determination on the population of microbe cell was conducted via a direct count method using hemacytometer to assess the chambers. This was carried out after the microbe culture was centrifuged for 30 days. The determination of total petroleum hydrocarbon (TPH) level used the gravimetric method. The phosphorus-dissolving test was carried out by growing the cell of microbes in a Petri dish using Pikovskaya media. Then, the halo zone was measured with a ruler.

Data analysis

The data was analyzed based on data scoring system.

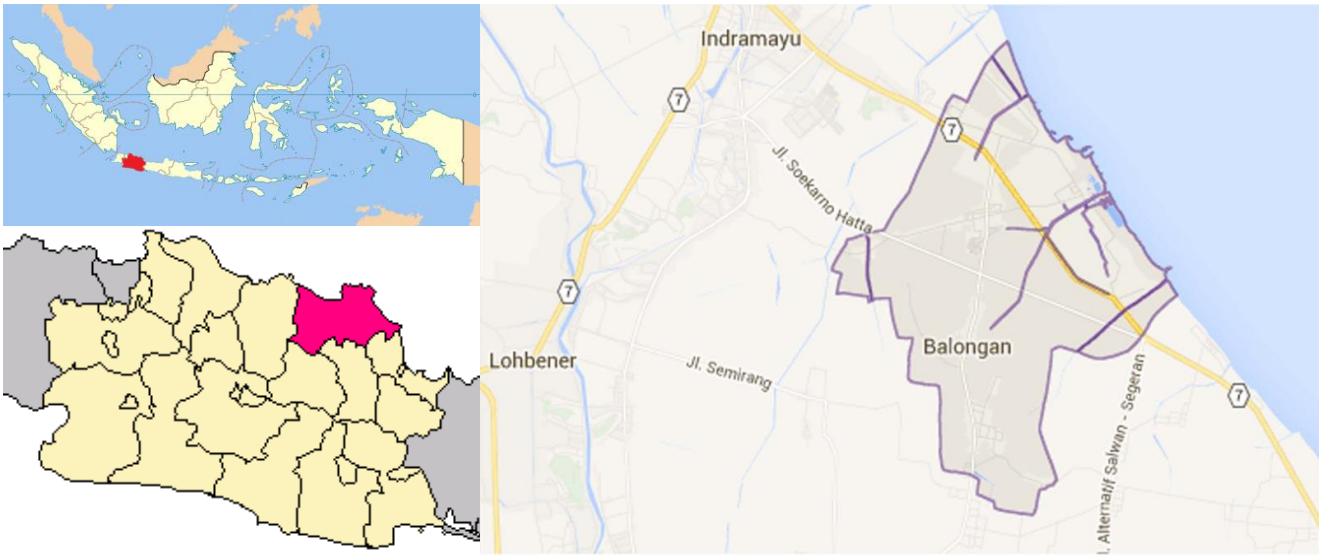

Figure 1. Location of Crude Oil Source from PT. Pertamina RU VI, Balongan-Indramayu, West Java, Indonesia.

RESULTS AND DISCUSSION

Total population of microbes cell

The result of this study showed that there is a difference in cell population level after the process of hydrocarbon degradation test for 30 days in different media with concentration of crude oil 1%. Additionally, each microbe from the 6 species were different from each other (Table 1). *Acinetobacter* sp. had a higher total population (34 x 107 and 140 x 108 CFU/gram) than *Azospirillum* sp. (166 x 105 and 167.5 x 106 CFU/gram). *B. subtilis* (49.5 x 107 and 115.7 x 108) had a higher cell population than *P. cepacia* (200 x 105 and 229 x 106 CFU/gram). *Penicillium* sp. had a higher cell population (378 x 103 and 115.8 x 104 CFU/gram) than *A. niger* (39.8 x103 and 56 x 104 CFU/gram). The determination for microbe cell population density was carried out with total plate count (TPC).

The efficiency of degradation of hydrocarbon

Based on the results of degradation of hydrocarbon on Table 2, the assertion of 6 microbes was carried out. It showed that *Acinetobacter* sp. (efficiency 93,40%) was more efficient than *Azospirillum* sp. (86,50%), then *P. cepacia* (99%) was more efficient than *B. subtilis* (84%), then *Penicillium* sp. (100%) was more efficient than *A. niger* (90,90%). The determination for hydrocarbon degradation measurement was conducted by determining hydrocarbon concentrate (TPH) for each treatment, then the value of TPH's efficient value was counted from each of TPH value.

Selection of microbes on the stage of hydrocarbon degradation test was carried out to know the capability of each microorganism in degrading crude oil hydrocarbon, until it became a comparison from microbe cell number in media test. Each microbe had a different capability in utilizing hydrocarbon as sole carbon source or substrate. The diversity of hydrocarbon forms, such as aliphatic and aromatic, can be degraded by specific microbes.

The potentiality to dissolve the phosphorus compounds

In addition, the cell population level and hydrocarbon degradation test needed to be tested. This was true especially of the phosphorus dissolving microbe groups, to know that they were able to dissolve the phosphorus substance in crude oil contaminated media, marked by a halo zone width in petri dish. Based on the results shown in Table 3, *P. cepacia* (0.16 mm) had a larger halo zone than *B. subtilis* (0.14 mm) for bacterial group. Then, for fungi group, *Penicillium* sp. (0.27 mm) had a larger halo zone than *A. niger* (0.18 mm).

Table 2. The efficiency of hydrocarbon degradation by soil microorganisms.

Microorganisms	**Efficiency of degradation Hydrocarbon (%)**
Nitrogen Fixation Microbes	
Acinetobacter sp.	93.40
Azospirillum sp.	86.50
Phosphorus Dissolved Bacteria	
Bacillus subtilis	84.00
Pseudomonas cepacia	99.00
Phosphorus Dissolved Fungi	
Aspergillus niger.	90.90
Penicillium sp.	100.00

Table 3. The result of phosphorus-dissolving microbes.

Microorganisms	**Halo zone Wide (mm)**
Phosphorus Dissolved Bacteria	
Bacillus subtilis	0.14
Pseudomonas cepacia	0.16
Phosphorus Dissolved Fungi	
Aspergillus niger	0.18
Penicillium sp.	0.27

Table 1. Total population of soil microorganisms.

Microorganisms	**TPC (CFU/gram)**					
	10^3	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**
Nitrogen Fixation Microbes						
Acinetobacter sp.	-	-	-	-	34	140
Azospirillum sp.			166	167.5	-	-
Phosphorus Dissolved Bacteria						
Pseudomonas cepacia	-	-	200	229	-	-
Bacillus subtilis	-	-	-	-	49.5	115.7
Phosphorus Dissolved Fungi						
Penicillium sp.	378.3	115.8	-	-	-	-
Aspergillus niger.	39.8	56	-	-	-	-

Discussion

The level of microbe cell population density is affected by microorganism enzymatic process in crude oil contaminated media, and the availability of sole carbon source also impresses the addition of microbe cells (Cookson 1995). Sufficient availability of a sole carbon source will increase the population cell. *Acinetobacter* sp. had higher total population than *Azospirillum* sp. It showed that *Acinetobacter* sp. had the best capability of metabolism. Furthermore, it has ability to survive in contaminated media. Jones et al (1983) reported that microbe is frequently found in site or in soil that is contaminated by dangerous and toxic waste, such as crude oil contamination (Jones et al 1983). According to Das and Chandran (2011), *Acinetobacter* sp. was known to be able to utilize the hydrocarbon of n-alkane, that had C10-C40 chain, as sole carbon source and energy. In literature, there was limited research discussing *Azospirillum* sp. in bioremediation process for crude oil. However, both *Acinetobacter* sp. and *Azospirillum* sp. were classified as plant growth-promoting rhizobacteria (PGPR) that was associated with plant root in phytoremediation process (Gałązka et al. 2012).

As phosphorus-dissolving microbes, *B. subtilis* had a higher population cell than *P. cepacia*. Then, *Penicillium* sp. had a higher population cell then *A. niger*. A previous study reported that *B. subtilis* could be found in some crude oil contamination sites and it still enhanced its population cell (Toledo et al 2006). In this study, *B. subtilis* was able to use the hydrocarbon of crude oil as a sole carbon source more than *Pseudomonas cepacea*.

Fungi is known to be the best degrading agent for hydrocarbon crude oil in the bioremediation process, compared to bacteria such *Penicillium* sp. and *A. niger*. Many studies have revealed that *A. niger* has higher activities in degrading hydrocarbon than *Penicillium* sp. (Al-Nasrawi 2012). However, this experiment showed that *Penicillium* sp. Had higher activities than *A. niger*, this can be seen from its high population.

Besides the cell population, another criterion for measuring the microbes' capability in bioremediation system is the efficiency of hydrocarbon degradation. Bacteria of *P. cepacia* had the capability to degrade aromatic hydrocarbon, while *B. subtilis* was able to degrade aliphatic hydrocarbon (Ghazali et al. 2004). A characteristic of a carbon chain for aromatic species is recalcitrant in the environment, until the value of efficiency of *P. cepacia* was higher than *B. subtilis*. Therefore, it has been shown that *P. cepacia* was more efficient that other bacteria in bioremediation process.

Acinetobacter sp. and *Azospirillum* sp. were PGPR microbes (plant growth-promoting rhizobacteria). Research has revealed that both microbes have potential in bioremediation application (Huang et al. 2004). *Acinetobacter* sp. Had capability to degrade the hydrocarbon (Ellis 1994; Johnsen et al. 2005) and able to enhance rhizoremediation process in soil contamination (Bhattacharyya and Jha 2011; Pajuelo et al. 2011; Vershinina 2012). Both *Acinetobacter* sp. And *Azospirillum* sp. Were able to use the hydrocarbon as a sole carbon source and energy. However, in this study, *Acinetobacter* sp. had a higher level of efficiency than *Azospirillum* sp. in the hydrocarbon degradation process.

In relation to the efficiency of hydrocarbon degradation in fungi group, it showed that *Penicillium* sp. was more efficient than *A. niger*. Also, if compared to bacteria, the fungi group had a higher efficiency. The fungi were better than bacteria as a biological agent on hydrocarbon biodegradation process (Vanishree et al. 2014). Both of these fungi were known to be able to utilize crude oil as substrate affected from extracellular enzyme that were produced by them (Adekunle and Adebambo 2007). In addition, both had potential to act as hydrocarbon degrading agent of crude oil. However, the superiority of *Penicillium* sp. toward *A. niger* was supported by its capability to survive in a crude oil contamination situation, even in the saline soil that *Penicillium* sp. could survive while others could not (Vanishree et al. 2014). So, *Penicillium* sp. is more potential than *A. niger*.

The last criteria of the four microbes was their function as phosphorus dissolving microbes namely *P. cepacia* (*Burkholderia cepacia*) (Holmes et al. 1998), *B. subtilis*, *Penicillium* sp. and *A. niger*. The test on the capability of the microbes group in dissolving phosphorus substance on Pikovskaya aimed to know their other function, for example, as biofertilizer. In a bioremediation system, the nutrient is one of the most important factors needed to manage the continuity of bioremediation process. So, it can work better in the mineralization process by microbes. One of them is capable of dissolving phosphate substance. The availability of nutrients in the soil was a primary factor in bioremediation process (Alvarez and Illman 2006). Capability to dissolve the phosphate is also determined by carbon source and energy for hydrocarbon degradation process.

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In vitro response of *Phomopsis theae* to the products of *Azadirachta indica* and extracts of *Warburgia ugandensis*

CHEPKORIR SOY LINNER[1], JONAH K. BIRGEN[2,♥], JOHN MAINGI[3]
[1]School of Pure and Applied Sciences, Kenyatta University. Nairobi, Kenya
[2]Department of Plant Sciences, Kenyatta University. Nairobi, Kenya. ♥email: jkbirgen2000@yahoo.co.uk
[3]Department of Microbiology, Kenyatta University. Nairobi, Kenya

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Abstract. *Linner CS, Birgen JK, Maingi J. 2017. In vitro response of* Phomopsis theae *to the products of* Azadirachta indica *and extracts of* Warburgia ugandensis. *Bioteknologi 14: 37-46.* This study was done to determine the use of plant products and extracts to manage the disease caused by *Phomopsis theae*. Among the products used were Nimbecidine and Trilogy, which are products of the neem tree. The *Warburgia ugandensis* extracts from the bark, root and leaf were also used *in vitro* to determine the inhibition of *Phomopsis theae*, the causative agent of Branch and Collar Canker. These were compared with the inhibition of standard fungicides (Topsin and Saaf). Nimbecidine and Trilogy were tested at the concentration of 10 ppm, 25 ppm, 50 ppm and 100 ppm. Additionally, *W. ugandensis* extracts were tested at the rates of 10 g/100 mL, 15 g/100 mL and 20 g/100 mL. Nimbecidine inhibited growth more than Trilogy in all the concentrations and were not significantly different from those of Topsin and Saaf. Stem bark extracts of *W. ugandensis* were also effective in inhibiting the growth of *P. theae* with inhibition of 97.64 per cent in all the rates. Root was next in inhibition with 78.8, 19.45 and 9.89 per cent in 20 g, 15 g, and 10 g respectively. The leaf extracts did not inhibit growth at any rate. In liquid media, similar results were observed. In Nimbecidine, mycelial weights were significantly lower compared to Trilogy. Stem bark extracts also had lower mycelial weights, followed by the root, and then leaf among the extracts. Nimbecidine and bark extracts of *W. ugandensis* were compared with standard fungicides, Topsin and Saaf, and the extract was comparable to the fungicides both in solid and liquid media. They were able to inhibit the growth of *P. theae.* It was concluded that Nimbecide and the bark extracts from *W. ugandensis* are potential alternatives, or can supplement the standard fungicides in the control of *Phomopsis theae* in tea.

Keywords: *Azadirachta indica, extracts product,* in vitro, *Phomopsis theae, Warburgia ugandensis*

INTRODUCTION

The history of tea (*Camellia sinensis* Kuntze (L.) in Kenya can be traced back to 1903 when G.W. Caine, a European settler, introduced the first seed and planted it in Limuru near Nairobi (TBK 2010). Tea is produced by smallholder farmers and large plantation such as Brooke Bond, African Highlands and Eastern Produce Limited. The large plantations are grouped in the Kenya Tea Growers Association (K.T.G.A.) and cover about 40% of Kenya tea production. Tea is an important income producer for many countries in the world. For example, tea annually contributes about 26% of Kenya's export earnings and 4% of Gross Domestic Product (GDP) (Wachira and Ronno 2004). In 2007, Kenya exported more than 360 million kilograms of processed tea that gave income to the country Kshs. 43 billion in the form of foreign exchange (TBK 2008). Since the introduction of tea in Kenya, the plantation areas have continued to grow that it has reached 149,196 hectares in 2007 (TBK 2007). This sector offers year-round jobs to approximately 639,521 farmers in rural areas, as well as providing employment in other tea-handling processes. As a labor-intensive industry, the tea sector supports the livelihood of over 3 million people directly and indirectly (TBK 2008).

The tea growing areas of Kenya are found in the Great Rift Valley. In the East Rift is the cool Arberdare plateau and Nyabene hill. While the West Rift is decorated by the cliffs Mau is the hill of Nandi, the highlands around Kericho, Mt. Elgon and the Kisii plateau. The slopes of this plateau are 1,500 m to 2,700 m above sea level and is where tea is planted. These areas are endued with an ideal climate for tea growth that include tropical and volcanic soils, which are rich in nutrients give a unique taste and character on the produced tea. Rainfall ranges from 1200 mm to 2700 mm per year (TRFK 2001).

Despite its importance, tea production encounters several challenges; these include the cost of tea production (labor, fuel, and electricity), mismanagement, tea-age bushes, high overhead costs, poor farming practices, climate change and shoddy roads that impede the rapid and efficient transportation of tea to the market. Fragmentation of tea farms in the face of declining global prices and rising production costs, threaten the survival of small-scale Kenyan tea farmers (Mburu 2008). Apart from all the challenges stated previously, there are also disease problems. The main diseases in Africa are the roots of Amillaria (*Amillaria heiminii*) and the wood rot (*Hypoxylon serpens*) (Onsando et al. 1997). Others including Branch and Collar Canker (*Phomopsis theae* (*P. theae*), Brown Blight (*Colletotrichum coccodes*) and Gray Blight (*Pestaliopsis theae*) are very important (Anon 1991). The losses in terms of quantity due to branch and collar canker have not been counted, even though the numbers are

large (Otieno 1998). Pathogens affect carbohydrate metabolism; they also cause rosette loss and accrued uniqueness due to excessive anthocyanin accumulation. Despite its economic impact, effective preventive measures are not available, in addition to healthy wood pruning and the application of copper fungicides to prune cutting (Ponmurugan et al. 2006). Random and extreme usage of chemical fungicides for seed and soil management have led to accrued pathogen resistance (Daghman et al. 2006). For example, benzimidazole fungicides are very effective in controlling the Gray Blight of tea, but fungicide resistant isolates have appeared in most of the cultivated areas in Kenya.

Trilogy (cleansed neem oil) is known to have antifungal activity (Moline and Lock 1993). According to Mirza et al. (2000), neem products are found to be very effective at various phytophthora infestants stages. Kazmi et al. (1995), reported that 0.1% of neem oil causes a significant decrement on growth of *Alternaria alternata* and *Aspergillus* spp. Locke (1995) reported that in the field, *A. alternata, Aspergillus niger* and *Fusarium oxysporum* were completely controlled by using 2-10 % of neem oil. According to Niaz and Kazmi (2005), neem oil is quite effective against *Aspergillus* spp. There was a need to check the effectiveness of neem oil on *Phomopsis theae*, as such a study has never been done before.

The objectives of this study were (i) to evaluate the effects of neem products (Trilogy and Nimbecidine) on the growth of *P. theae*; (ii) to evaluate the effects of bark, root, and leaves extract of *W. ugandensis* on the growth of *P. theae*; (iii) to compare the effects of neem products and *W. ugandensis* extract on the growth of *P. theae* with standard Topsin and Saaf fungicide.

MATERIALS AND METHODS

Study site

The study was conducted in Ainamoi Village, Kericho District, Kenya (Figure 1). The study was conducted at the laboratory of Tea Research Foundation in Kenya (TRFK) in Kericho District. Kericho District is in the valley of Lake Victoria. Its geology is characterized by volcanic and metamorphic complexes. The district receives rainfall with a low evaporation rate. Temperatures range from 10°C to 29°C and rainfall patterns are so unique that the center of tea growing receives the highest rainfall of approximately 2125 mm (TRFK 2001). The soil is slightly acidic, with a pH of 5.5 which favors the growth of tea and coffee. The soil is muddy but has been well drained and has most of the nutrients needed for plant growth.

Neem products and standard fungicides source

Nimbecidine (azidarachtin) and Trilogy (hydrophobic neem extract) were neem products. Nimbecidine (azidarachtin) was manufactured by T. Stanes and Company Limited in India, while Trilogy (hydrophobic neem extract) was manufactured by Certis Company in the USA. They were purchased from Paksons Agrochemical shop in Kericho. Standard fungicides (Saaf and Topsin) were obtained from the pesticides store in TRFK in Kericho.

Culture source and inoculum preparation

One of pathogen cultures is *Phomopsis theae*. It was acquired from the stock kept at TRFK in Kericho. *P. theae* mycelium plugs were acquired aseptically from PDA agar slants of stock cultures using a flame sterilized needle and placed to the center of PDA media in 9 cm diameter petri dish. One piece was placed on each plate and incubated at 18-22°C for 7 days. The inoculation was performed in a sterile lamina flow hood for aseptic conditions.

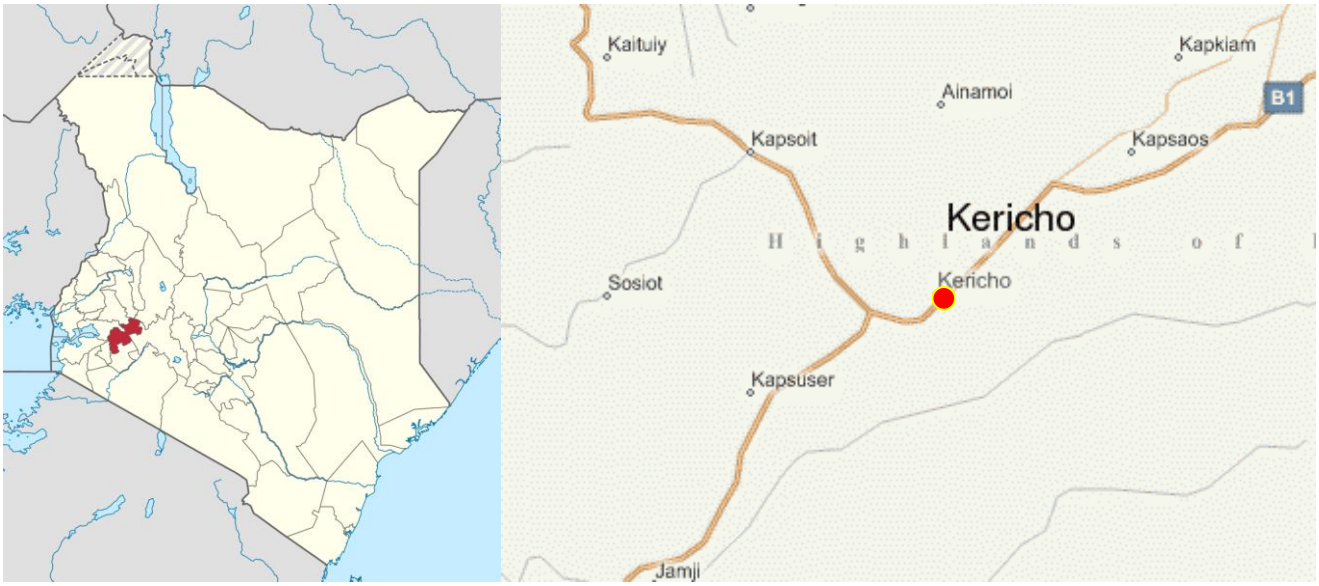


Figure 1. Map of Kenya showing location of Kericho District.

Efficacy of neem products on *Phomopsis theae* in solid media: inoculations using agar dilution method

3.90 g of PDA were liquefied in each of the seventeen 250 mL conical flasks containing 100 mL of distilled water and sterilized by autoclaving at 121ºC for 20 minutes at 15 psi. The fungicides were then incorporated into the medium at the rate of 0.05 mL, 0.13 mL, 0.25 mL, and 0.50 mL for Nimbecidine and 0.53 mL, 1.33 mL, 2.67 mL, and 5.33 mL for Trilogy to make 10, 25, 50, and 100 ppm respectively.

The amended media in each Petri dish was centrally injected with 2 mm mycelia discs cut from margins of three-day old cultures of *P. theae* growing on PDA medium, using flame sterilized cork borer. The Petri dishes, which contained unamended PDA, acted as controls. The inoculated Petri dishes were arranged in a completely randomized design with four replications on a laboratory bench. Growth of the pathogen (*P. theae*) on the amended and unamended media (controls) was assessed by measuring mycelial diameter of the pathogen at three-day interval for a period of 30 days.

Efficacy of neem products on *Phomopsis theae* in liquid media: inoculation in malt extract liquid media

Eighteen grams (18 g) of malt extract were weighed one by one using analytical balance and dissolved in 1000 mL conical flasks containing 600 mL of sterilized water with 0.3 g of peptone. The neem products were incorporated into the media at 3.36 mL, 6.72 mL, 13.40 mL, and 26.80 mL for Nimbecidine and at 0.08 mL, 2.10 mL, 4.30 mL, and 8.95 mL for Trilogy and were also sterilized at 121 ºC for twenty minutes at 15 *psi* using an autoclave. The conical flasks without the products and fungicides were set up. They were agitated thoroughly using a magnetic stirrer hot plate 400 (Gallen kamp) at 500 revolutions per minute. 150 mL of each medium were measured using a sterile measuring cylinder into 250 mL conical flasks.

A method described by Onsando (1988) to study morphology of *Armillaria mellea* was used. Mycelia from culture of *A. mellea* on MEA was used to inject petri dishes of agar. A sterile loop was used to excise 3-5 mm mycelia that were placed into the center of a Petri dish. Cultures were incubated at 25 ºC for 3-6 weeks. Inoculation with the P228 isolate was carried out in a lamina flow hood. Using a sterile 2 mm diameter cork borer, mycelial agar discs were cut from the margins of colonies of three-day culture of *P. theae* isolate. Four discs were placed in each conical flask using a sterile inoculating needle. The needle was sterilized by dipping it in 70 % ethanol and flaming on a Bunsen burner. The conical flasks were incubated at room temperature on an orbital shaker (Gallen kamp SGM 300 SLUO) at 40 revolutions per minute for three weeks for the purpose of aeration. To obtain dry fungal biomass, the fungal biomass was harvested using Whatman's filter paper previously dried to constant weight. The harvested mycelium along with the filter paper were dried to constant weight and the weight of each fungal biomass determined.

In vitro* screening of stem bark, root, and leaf extracts of *Warburgia ugandensis* for their antifungal properties against *Phomopsis theae

Screening using agar diffusion method

Warburgia ugandensis samples were chopped into small pieces. Ten, fifteen and twenty grams of leaves, bark and roots were weighed separately and put into 250 mL Pyrex conical flasks which contained 150 mL de-ionized water. The samples were infused by autoclaving at 121ºC for 20 minutes at 15 psi using an autoclave (Gallen Kamp). Thereafter, filtration was done, and 150 mL of the filtrate transferred into 250 mL conical flask containing 5.85 g plain agar. These were then sterilized by autoclaving at a temperature of 121ºC for 20 minutes. Measurements of 20 mL of warm autoclaved agar-infusion mixture were poured into each of 9 cm diameter sterile plastic disposable Petri plates (BS 611) in a lamina flow hood. Two-millimeter diameter mycelia agar disks were cut from the margins of 7-10 days old colonies of the *P. theae* isolates cultures using a 2 mm diameter cork borer. The discs were centrally placed using a transfer needle into the 9 cm diameter plastic Petri dishes containing the leaf-agar, root-agar and stem bark-agar infusion and sealed with parafilm. Three leaf extracts, three stem bark extracts and three root extracts constitute nine treatments, with plain agar served as a control. All treatments were replicated four times in a completely randomized design and incubated at room temperature. The radial colony diameter was measured at an interval of 48 hours for a period of three weeks.

Screening in liquid media

A method described by Onsando (1987) was used. Sixty grams of fresh leaves stem barks and roots of *Warburgia ugandensi*s were transferred into 1000 mL sterilized labeled Pyrex conical flasks containing 600 mL of de-ionized water. The leaves, stem bark and roots were infused at 121ºC for 20 minutes at 15 psi. Using a sterile measuring cylinder, 150 mL infusion of the leaves, bark and roots were filtered aseptically with a sterilized glass funnel plugged with a small cotton wool into four 250 mL Pyrex conical flasks to make four replicates. A different funnel was used for each to avoid contamination. Inoculation, incubation, and harvesting were carried out as described in the previous experiment.

Harvesting of mycelium

Fungal mats were harvested after 3 weeks by filtering using Whatman`s filter paper previously dried to constant weight and weighed together with agar blocks that formed part of the inoculums. The harvested mycelia along with the filter paper were dried in an oven (Menimen) at 60 ºC to constant weight. The weight of each fungal biomass plus the filter paper were measured using an analytical balance. The dry weight of each biomass was determined.

Effect on growth of *Phomopsis theae*

Effect of neem product, standard fungicides and Warburgia ugandensis *extracts on growth of* Phomopsis theae *using agar diffusion method*

1.5 g of PDA was liquified in 100 mL of de-ionized water in 250 mL Pyrex conical flasks and sterilized by autoclaving at 121 ºC for 20 minutes at 15 psi. The most active neem product (Nimbecidine) was incorporated into the medium at different rates to make 10, 25, 50, and 100 ppm. The fungicides were also incorporated into the medium at 0.05 mL, 0.13 mL, 0.25 mL, and 0.50 mL for Topsin and 0.53 mL, 1.33 mL, 2.60 mL, and 5.33 mL for Saaf to make 10, 25, 50 and 100 ppm respectively. The most effective of the *W. ugandensis* (bark) samples were chopped into small pieces, and then 10, 15 and 20 g weighed and put into 250 mL Pyrex conical flask containing 150 mL of de-ionized water. They were then infused at 121 ºC for 20 minutes at 15 psi. The infusion was filtered, and 100 mL transferred into 250 mL conical flask containing 1.5 g of plain agar and autoclaved at 121 ºC for 20 minutes. The agar-infusion, neem product-medium and fungicides-medium mixtures were agitated thoroughly using a magnetic stirrer hot plate, at 500 revolutions per minute. Approximately 20 mL of the mixture were poured into each of 9 cm diameter sterile disposable Petri dishes in a lamina flow hood. Inoculation, incubation, and harvesting were done as in the previous experiment.

Effect of neem product, standard fungicide and Warburgia ugandensis *extract on growth of* Phomopsis theae u*sing broth dilution method*

Eighteen grams of malt were weighed separately using analytical balance then dissolved in sixteen conical flasks containing 600 mL of sterilized water with 0.3 g of the peptone. The most active neem product (Nimbecidine) and fungicides (Saaf and Topsin) were incorporated as in previous section, while the most effective *W. ugandensis* extract (bark) was incorporated as in previous section. Inoculation, incubation, and harvesting were done as in the previous sections.

Data analysis

All the results were subjected to Analysis of Variance (ANOVA) using GMSTAT software to test for significant difference in radial growth and mycelial weights of *P. theae*, among the standard fungicides (Topsin and Saaf), neem products (Nimbecidine and Trilogy), *Warburgia ugandensis* extracts (leaves, bark, and roots) and control. Separation of means was carried out using Tukeys` test at P≤0.05.

RESULTS AND DISCUSSION

***In vitro* efficacy of neem products (Trilogy and Nimbecidine) on growth of *Phomopsis theae* in solid media**

For Nimbecidine, a neem product, the radial measurements in 10 ppm, were 2.00 mm, 3.50 mm, 7.00 mm, and 12.50 mm for day 6, 15, 21 and 30 respectively. At the concentration of 25 ppm, the measurements were 2.00 mm, 2.00 mm, 7.25 mm and 10. 00 mm for day 6, 15, 21 and 30 respectively. The measurements in day 6, 15, 21 and 30 were 2.00 mm, 2.00mm, 3.00 mm and 4.75 mm. while in 100 ppm, they were 2.00 mm for day 6, 15 and 21 and 3.56 mm in day 30 (Table 1). In Trilogy, the radial measurements were 2.00 mm, 8.73 mm, 39.50 mm, and 62.00 mm in the concentration of 10 ppm, for day 6, 15, 21 and 30 respectively. In 25 ppm, the measurements 2.00 mm, 7.25 mm, 38.25 mm, and 56.25 mm in the days of the experiment. In the concentration of 50 ppm, the measurements were 2.00 mm, 4.00 mm, 10.25 mm, and 35.00 mm in 100 ppm (Table 1).

At the lower concentration, 10 ppm, all the treatments were remarkably similar (P≤ 0.05, df=79) on day 6 and 15. However, on day 21, Trilogy was seen to differ remarkably from Nimbecidine (Table 1). At 25 ppm, no significant difference was seen on day 6 in various treatments. On day 15, there was no significant difference between Trilogy and control (P≤ 0.05, df= 79), while there was remarkably different between Trilogy and Nimbecidine. On day 21, the measurements for radial growth of *P. theae* in media rectified with Nimbecidine and Trilogy were 7.25 mm and 38.25 mm, respectively. Trilogy was seen to be remarkably different from Nimbecidine treatment.

In the concentration of 50 ppm, all the treatments did not differ from each other. Similarly, on day 15, there was no significant differences (P≤0.05) among the treatments. However, on day 21 and 30 Trilogy differed from Nimbecidine treatment (Table 1). In 100 ppm, no significant differences were noted on days 6 and 15 in all the treatments (P≤0.05). On days 21 and 30, radial measurements of *P. theae* in media rectified with Trilogy were 9.00 mm and 27.00 mm. These were remarkably superior (P≤ 0.05, df=79) to that in media rectified with Nimbecidine which were 2.00 mm and 3.56 mm, respectively. In the media rectified with Nimbecidine and Trilogy, there was higher percentage of impediment compared to that of the unrectified media (control) (Figure 2, Table 1).

Impediment of fungal growth by Nimbedicine was accrued with the accruement in concentration hence rates of impediment of 95.33, 93.77, 86.6 and 83.67, at 100, 50, 25 and 10 ppm respectively were recorded. Comparatively, Trilogy also inhibited growth though in small percentages (19, 56.26, 53.60 and 49.50) unlike in Nimbecidine. Percentage impediment also accrued with accruement in the concentrations of 10 ppm, 25 ppm, 50 ppm and 100 ppm (Table 1).

Efficacy of neem products on growth of *Phomopsis theae* in liquid media

Among the neem products, higher mycelial weights were recorded in Trilogy compared to Nimbecidine. Among the various concentrations of Trilogy tested, the weights recorded were 1.47 g, 1.45 g, 1.20 g, and 0.87 g in 10 ppm, 25 ppm, 50 ppm and 100 ppm respectively (Table 2). In the media rectified with Nimbecidine, mycelial weights of 0.72 g, 0.66 g, 0.61 g, and 0.43 g in 10, 25, 50 and 100 ppm were recorded though they were not

remarkably different (P≤ 0.05) from each other (Table 2). At the lower concentration (10 ppm), Nimbecidine remarkably suppressed the growth of the pathogen compared to Trilogy, though the later was seen to inhibit growth more than in the control. In the concentration of 25 ppm, Trilogy was remarkably higher (P≤ 0.05) than those of Nimbecidine and remarkably lower than the control. In the concentration of 50 ppm of Nimbecidine and Trilogy, were 0.61 g and 1.20 g respectively (Table 2). The mycelial weights in Nimbecidine were remarkably lower (P≤ 0.05) than in Trilogy and in the control. At a higher concentration (100 ppm), Nimbecidine and Trilogy were remarkably different (P≤ 0.05) from each other. Nimbecidine inhibited growth more than Trilogy but were not remarkably different from (P≤ 0.05) each other but from the control. In all the concentrations, Nimbecidine was seen to remarkably suppress (P≤ 0.05) the growth of the pathogen compared to Trilogy (Table 2).

Efficacy of the *Warburgia ugandensis* extracts (leaf, root, and stem bark) on the growth of *Phomopsis theae* in solid media

The radial growth of *P. theae* in the media rectified with bark extracts was 2.00 mm in all the rates throughout the experimental period. However, on days 6 and 15, the radial measurements were not remarkably different from the other extracts. On days 21 and 30, radial measurements in the bark extracts were remarkably lower (P≤ 0.05) than that of the root and leaf extracts (Table 3). The media rectified with root extracts, also showed impediment of the growth of the pathogen.

On day 6, the radial measurements were 2. 00 mm for the three rates 10 g, 15 g, and 20 g (Table 3). On day 15, radial measurements were 8.70 mm, 6.80 mm and 5.00 mm and were not remarkably different (P≤ 0.05) from each other. For day 21, the measurements were 33.30 mm, 28.80 mm, and 9.70 mm in 10 g, 15 g, and 20 g respectively.

There was no significant difference (P≤ 0.05) between the lower rates 10 g and 15 g, but were different from the higher rate (20 g). On day 30, radial measurements were 66.00 mm, 59. 00 mm and 15. 00 mm, for 10 g, 15 g, and 20 g respectively.

There was significant difference (P≤ 0.05) among the three rates, with the higher rate being remarkably lower than the other rates. All the rates showed significant difference (P≤ 0.05) from that of the control. In the media

Table 1. Radial measurements and percentage impediment of *Phomopsis theae* growing on media rectified with neem products (Trilogy and Nimbecidine) on Days 6, 15, 21 and 30 after Treatment.

Treatments		Mean radial measurements of *Phomopsis theae* (mm) Number of Days				% impedi-ment
		6	15	21	30	
Trilogy	10 ppm	2.00a[1]	8.73ab	39.50a	62.00b	19.00
	25 ppm	2.00a	7.25ab	38.25a	56.25c	56.26
	50 ppm	2.00a	4.00ab	10.25b	35.50b	53.60
	100 ppm	2.00a	4.00ab	9.00b	27.00c	49.50
Nimbe cidine	10 ppm	2.00a	3.50ab	7.00bc	12.50f	83.67
	25 ppm	2.00a	2.00b	7.25cd	10.00f	86.60
	50 ppm	2.00a	2.00b	3.00c	4.75g	93.77
	100 ppm	2.00a	2.00b	2.00c	3.56g	95.33
Control		2.00a	12.50a	41.50a	76.25a	0.00

Note: [1]Mean values in the same column followed by similar letters are not remarkably different at P≤0.05

Table 2. Mycelial weights of *Phomopsis theae* in liquid media amended with neem products (Nimbecidine and Trilogy**).**

Treatment	Mycelial weights in grammes at different concentrations			
	Concentration (ppm)			
	10	25	50	100
Trilogy	1.47b[1]	1.45b	1.20bc	0.87cd
Nimbecidine	0.72de	0.66de	0.61de	0.43de
Control	2.16a	2.16a	2.16a	2.16a

Note: [1]Mean values in the same column followed by similar letters are not remarkably different at P≤0.05

A **B** **C**

Figure 2. *Phomopsis theae* growing in media rectified with Neem products (Nimbecidine and Trilogy) and control. A. Nimbecidine, B. Trilogy, C. Control.

rectified with leaf extracts, the radial measurements on day 6 were 2.00 mm in each of the rates, 10 g, 15 g and 20. They were not remarkably different (P≤ 0.05) from each other. On day 15, the radial measurements were 11.30 mm, 12. 50 mm and 13,00 mm in 10 g, 15 g, and 20 g respectively (Table 3). There was no significant difference (P≤ 0.05) among them. For day 21, radial measurements were 55.80 mm, 54.00 mm and 47.80 mm in 10 g, 15 g, and 20 g. the lower rate was remarkably different (P≤ 0.05) from the higher rate. All the rates were remarkably lower (P≤ 0.05) than that of the control. On day 30, in the three rates had radial diameter was 85.00 mm and were remarkably higher (P≤ 0.05) than the control.

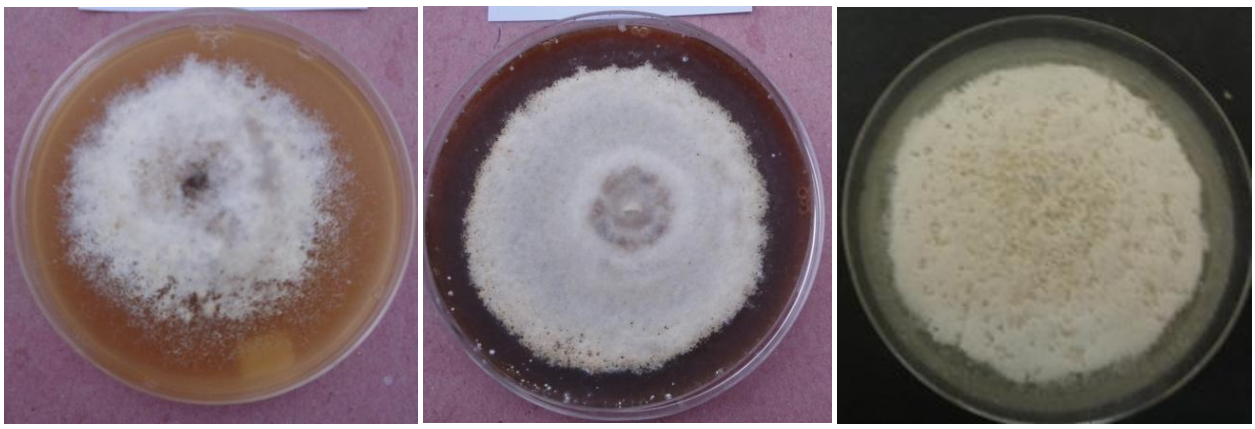
At the lower rate (10 g), there was no significant difference among the radial measurements in all the extracts, bark, root, and leaf. On days 6 and 15, they were not remarkably different (P≤ 0.05) from that in the control (Table 3). On day 21, the radial measurements in the media rectified with bark extracts were significantly lower (P≤ 0.05) than in media rectified with root, leaf extracts and in the control. The measurements in the root extracts were not remarkably different (P≤ 0.05) from that of the control. The radial measurements in the leaf extracts were remarkably superior among all the extracts. On day 30, the measurements in bark extracts were also remarkably lower (P≤ 0.05) than in the media rectified with the other extracts and in the control. The measurements in the root extracts were remarkably lower than in the leaf and control, which were remarkably different (P≤ 0.05) from each other.

In the media rectified with 15 g of the extracts, there were no significant difference among the measurements in the bark, root, and leaf extracts on days 6 and 15. On day 21, the mycelial measurements in the bark were remarkably lower (P≤ 0.05) than in the root extracts, leaf extracts and the control. Similarly, the radial measurements were remarkably lower (P≤ 0.05) than in the leaf extracts and the control. The measurements in the leaf extracts were remarkably higher (P≤ 0.05) than in the control (Table 3). On day 30, the radial measurements in the media rectified with leaf extracts were remarkably the highest (P≤ 0.05) among all the treatments while the measurements in the bark were the lowest. For the highest rate (20 g) of the extracts, the radial measurements on day 6 and 12 were not remarkably different (P≤ 0.05) from each other and that in the control. On day 21, the measurements in the root were remarkably lower (P≤ 0.05) than those in the leaf and control, and higher than the measurements in the bark extracts. On day 30, the measurements in the bark extracts were remarkably the highest among the extracts and control. The measurements in the leaf extracts were significant lower (P≤ 0.05), even compared to the control (Figure 3). The measurements in the media rectified with root extracts were remarkably lower than in the control.

Efficacy of *Warburgia ugandensis* extracts (leaf, root, and bark) on growth of *Phomopsis theae* in liquid media

In the media amended with bark extracts, there was complete impediment of growth of *P. theae;* hence the least mycelial weights recorded. In 10 g, 15 g and 20 g of the extracts mycelial weights were 0.02 g, which was remarkably lower than that of the other extracts and the control (Table 4). The root extracts also inhibited the pathogen to certain degree. The mycelial weights in 10 g, 15 g and 20 g were 0.95 g, 0.92 g, and 0.28 g respectively. The higher rate was remarkably different from that of 10 g and 15 g, and was not different from that of the bark. In the leaf extracts, mycelial weights in 10 g, 15 g and 20 g were 1.31 g, 1.19 g, and 1.0 g respectively. They were not remarkably different from each other.

The mycelial weights of *P. theae* in 10 g of the bark extracts was 0.02 g. While in the root and the leaf, it was 0.95 g and 1.34 g respectively. The mycelial weights in the root and the leaf were not remarkably different from each other. The bark differed from the root, leaf and the control. At the rate of 20 g, the mycelia weights of the pathogen were 0.02 g, 0.28 g and 1.0 g in media rectified with bark, root, and leaf extracts respectively. The mycelial weights in that of the bark and root did not differ remarkably from each other. Leaf extracts were remarkably higher than that of the root and bark extracts. Though the bark extracts had the lowest



A **B** **C**

Figure 3. *Phomopsis theae* growing on media rectified with *W. ugandensis* extracts and control. A. Root extracts, B. Leaf extracts, C. Control.

mycelial weights, the lower rate did not differ remarkably from the higher rate of the root extracts. Mycelial weights of the pathogen in the media rectified with 15 g and 20 g of the leaf extracts were not remarkably different from that of 10 g of the root extracts. The unrectified media had mycelial weight of 2.31 g and was remarkably different from that of the bark, root, and leaf extracts.

In vitro efficacy of the most effective neem product (Nimbecidine), standard fungicides (Topsin and Saaf) and the most effective *Warburgia ugandensis* extracts (bark) on growth of *Phomopsis theae*

In the media rectified with standard fungicides, Topsin and Saaf, the mean radial measurements of *P. theae* were 2.00 mm in all the concentrations (10 ppm,25 ppm, 50 ppm, 100 ppm) throughout the experimental days (6, 12, 24 and 30). These measurements were like those of the media rectified with bark extracts of *W. ugandensis.* The percentage impediment of the growth was 97.61 for Topsin, Saaf and bark extracts (Table 5).

Growth of *P. theae* in Nimbecidine was remarkably higher than in fungicides and bark extracts on days 12, 24 and 30, but was lower than in the control. The growth of the fungus in the higher concentration (50 ppm and 100 ppm), was remarkably lower than in the lower concentrations (10 ppm and 25 ppm) from day 12 to day 30 and lower than the growth in the control (Table 5). Growth in the control was the highest throughout the experiment, while in Topsin, Saaf and bark extracts, it was the lowest.

The impeding of growth impediment was highest in Topsin, Saaf and bark extracts but not different in percentage values. They were all inhibited at 97.61%. In Nimbecidine, growth impediment varied with the concentration, with the highest concentration impeding the growth at 94.33%, followed by 89.25% at 50 ppm, 85.67% at 25 ppm and 81.50% at 10 ppm. However, there was 0% impediment in the control (Table 5).

Table 3. Radial measurements of *Phomopsis theae* growing on media rectified with leaf root and bark extracts of *Warburgia ugandensis* on days 6, 15, 21 and 30.

Treatment	(g)	Mean radial mycelial measurements of *Phomopsis theae* (mm) Number of days				% impedi-ment
		6	15	21	30	
Bark	10	2.0a[1]	2.0a	2.0f	2.0f	97.27
	15	2.0a	2.0a	2.0f	2.0f	97.27
	20	2.0a	2.0a	2.0f	2.0f	97.27
Root	10	2.0a	6.8a	33.3cd	66.0c	9.96
	15	2.0a	8.7a	28.8d	59.0d	19.50
	20	2.0a	5.0a	9.7e	15.5e	78.85
Leaf	10	2.0a	11.3a	55.8a	85.0a	-15.96
	15	2.0a	12.5a	47.8b	85.0a	-15.96
	20	2.0a	13.0a	54.0a	85.0a	-15.96
Control		2.0a	12.5a	39.3c	73.3b	0

Note: [1]Mean values in the same column followed by similar letters are not remarkably different at P≤0.05. g-grams per 150 mL of water

Table 4. Mycelial weights of *P. theae* in liquid media amended with *Warburgia ugandensis* extracts, from the bark, root, and leaf at different rates.

Treatment	Mycelial weights in grammes in different rates of extracts Rates (g)		
	10	15	20
Bark	0.02d1	0.02d	0.02d
Root	0.95c	0.92c	0.28d
Leaf	1.34b	1.19bc	1.0bc
Control	2.31a	2.31a	2.31a

Note: [1]Mean values in the same column followed by similar letters are not remarkably different at P≤0.05. g-Grams per 150 mL of water

Table 5. Radial measurements of *Phomopsis theae* growing on media amended with standard fungicides (Topsin and Saaf), neem product, Nimbecidine, and *Warburgia ugandensis* Extract, (bark) on days 6, 12, 24 and 30.

Treatment		Means radial measurements of *Phomopsis theae* (mm) Number of Days				% impedi-ment
		6	12	24	30	
Topsin	10 ppm	2b[1]	2c	2d	2d	97.61
	25 ppm	2b	2c	2d	2d	97.61
	50 ppm	2b	2c	2d	2d	97.61
	100 ppm	2b	2c	2d	2d	97.61
Saaf	10 ppm	2b	2c	2d	2d	97.61
	25 ppm	2b	2c	2d	2d	97.61
	50 ppm	2b	2c	2d	2d	97.61
	100 ppm	2b	2c	2d	2d	97.61
Nimbeci	10 ppm	2b	7.25b	12.75b	15.5b	81.50
dine	25 ppm	2b	6b	10b	12b	85.67
	50 ppm	2b	3c	7.25c	9c	89.25
	100 ppm	2b	2c	4.25cd	4.75cd	94.33
Bark	10 ppm	2b	2c	2d	2d	97.61
	15 ppm	2b	2c	2d	2d	97.61
	20 ppm	2b	2c	2d	2d	97.61
Control	12.5a		29.75a	77a	83.75a	0

Note: [1]Mean values in the same column followed by similar letters are not remarkably different at P≤0.05

Table 6. Mycelial weights of *Phomopsis theae* grown in liquid media amended with neem products (Nimbecidine), standard fungicides (Topsin and Saaf) and *Warburgia ugandensis* (bark) extracts.

Treatment	Mycelial weights in grammes at different concentrations after three weeks of incubation Concentration (ppm)			
	10	25	50	100
Topsin	0.02c[1]	0.02c	0.02c	0.02c
Saaf	0.02c	0.02c	0.02c	0.02c
Nimbecidine	0.74b	0.66b	0.62b	0.45b
Bark	0.02c	0.02c	0.02c	0.02c
Control	2.31a	2.31a	2.31a	2.31a

Note: [1]Mean values in the same column followed by similar letters are not significantly different at P≤0.05

Efficacy of the most effective neem product (Nimbecidine), standard fungicides (Topsin and Saaf) and the most effective *Warburgia ugandensis* extracts (bark) in liquid media

Topsin, Saaf and bark extracts inhibited the growth in all the concentrations. Therefore, mycelial weights of 0.02 g were recorded after being incubated for 3 weeks. There was no considerable difference among the weights in these media. For Nimbecidine, the growth was better than that in the media amended by fungicides and bark extracts. There was an accruement in impediment, with an accruement in the concentration of the neem product. However, there was no considerable difference among them. Mycelial weights were considerably lower than the ones within the control in all the concentrations. The weights within the control were considerably highest in comparison to those within the amended plates (Table 6).

Discussion

Neem products efficacy (Nimbecidine, Trilogy) on Phomopsis theae *growth*

The observation found out that Nimbecidine drastically inhibited the growth of *P. theae*, and it was greater than the alternative neem product (Trilogy), both in solid and liquid media (table 1 and 2). Nimbecidine comprises compounds that have antifungal activity. These compounds might contain azadirachtin, meliantrol, slanin and nimbin. Dubey and Kumar (2009) pronounced that the fungicidal impact of azadirachtin was like the impact made by the fungicides bavistin and mancozeb. It has been shown to have considerably inhibited the growth of plant pathogenic fungi such as of *Fusarium oxysporum*, *Rhizoctonia solani*, *Altenaria solani* and *Sclerotina sclerotiorum* (Moslem 2009). The same consequences were observed by Moline and Locke (1993), who found out that neem oil had various fungicidal interests toward apple decaying fungi such as *Botryis cinera* ex. Fr, (grey mold) and *Glomerella cingulata* (anthracnose fungus). Moreover, Bohra et al. (2006) suggested that neem has active elements including azadirachtin, nimbin and nimbicin that are antifungal in nature. Research has shown that neem oil has negative effects on *Beauveria bassiana*, by inhibiting germination, colony diameter and conidiogenesis (Hirose et al. 2000). Different results by Babu et al. (2000) stated that spraying 3 % of neem oil onto tomato pot cultures resulted in 53 % reduction in ailment incidence compared to the control, while Patil et al. (2000), found out that incidence of tomato early blight because of *Alternaria solani* was lowered by neem oil with accrued fruit yield.

Trilogy, a miticide and agricultural fungicide, showed lower impediment of growth compared to Nimbecidine (Table 1 and 2) at the higher concentration (100 ppm), it had an impediment of 64.59 per cent in solid medium that is lower than other neem product (Table 1). Similar effects were also seen in the liquid media. The results are not in agreement with that of Poiloakidou (2005), who pronounced that Trilogy was powerful in opposition to Pseudomonas xanthii at lower concentrations in a laboratory assay. This could be due to the fact *P. xanthii* is a bacterium and therefore the difference could be caused by the mode of action of the plant products on fungi and bacteria. The result agrees with that reported by Wszelaki et al. (2002), who found out that Trilogy had no effect on early blight and *Septoria* leaf spot disease control that are caused by *Alternaria solani* and *Septoria lycopersici* respectively. Data that is available regarding the use of Trilogy indicates that Trilogy manages diseases in curcurbits to a limited extent (Meister 1999).

In different vegetation, Trilogy affords no ailment management, an observation sister to the placement depicted by the statistics recorded from the study (Tables 1 and 2). Neem oil failed to suppress spot anthracnose on leaves of dogwood (Hagan and Akridge 2007). Seaman et al. (2004), stated that Trilogy didn't suppress foliar illnesses caused by *A. solani* and *Septoria lycopersici*. Another investigator, Aboellil (2007) mentioned that a natural product from *A. indica*, Trilogy, inhibited many growth parameters of cucumber powdery mildew pathogen (Podosphaera xanthii) and induced resistance in cucumber plants. The activities difference of the two neem products could be due to their active ingredients. Nimbecidine has 0.03 % azadirachtin and Trilogy, that is hydrophobic extract, has less than 0.03 % azadirachtin.

Warburgia ugandensis *extracts efficacy on* Phomopsis theae *growth*

The fungicide properties of the bark agreed with the formerly conducted research. Numerous chemical and pharmacological studies conducted to observe extracts from the bark of Warburgia species have showed the presence of antifungal, antiulcer, insect antifeedant, molluscal, antimycobacterial and antiheshamanial active sesquiterpenes (Lunde and Kubo 2000; Wube et al. 2005; Ngure et al. 2009). Epipolygodial, mannilol, muzigadial, polygodial, tannin and warbuganal are examples of alkaloid group of metabolites present in the bark of *W. ugandensis* (Bekalo et al. 1996). A range of biological impacts consisting of trypanocidal, antiviral, fungicidal and antibacterial activity have been accredited to them.

Warburgia ugandensis extracts had been previously examined and found to show a vast sprectrum antimicrobial activity against quite a few microorganisms, including *Candida utilis*. It is additionally stated to have antimicrobial activity against *F. oxysporum*, *F. solani*, *Alternaria* spp., *R. stolonifer*, *A. niger*, *R. solanacearum* and *S. ipomoeae*, which are soil pathogens in association with rotting of sweet potato and other root crops (Ristaino 1993). This suggests that the pathogens may be controlled using herbal extracts, as had also been observed in different studies (Okigbo and Nmeka 2005).

Leaf extracts were least effective in impeding the accruement of the pathogen. In the 3 rates, radial growth was not remarkably distinct from each other. The results disagreed with those of Oniango (2003), who mentioned that leaf extracts of *W. ugandensis* gave most impeding of growth of *P. theae*, even within the lowest concentration. The distinction between the observation from the contemporary study and that of Oniango (2003) could be due to the solvents used for extraction which may not have captured all the active components from the plant extracts.

Olila et al. (2001), demonstrated that this plant has both antibacterial and antifungal activities. The outcomes show that the antifungal effects in *W. ugandensis* extracts are dependent on the core and part of the plant. This agrees with work done by Olila (1993). This can be due to the difference in concentrations of the various active metabolites in the different parts of the plant.

Efficacy of the most effective neem product (Nimbecidine), standard fungicides (Topsin and Saaf) and the most effective Warburgia ugandensis *extracts (bark) at the growth of* P. theae

The results of the study comparing the efficacy of neem product Nimbecidine, bark extracts and fungicides (Tables 5 and 6) indicated that bark extracts inhibited the growth of *P. theae* at all the rates. They inhibited the growth even at a lower concentration. The antifungal effects of *W. ugandensis* have been previously observed. A study in Kenya showed the extracts from the plant act against soil pathogens specifically; *Fusarium oxysporum*, *Alternaria passiflorae* and *Aspergillus niger* (Ruggutt et al. 2006).

Inside the neem product, Nimbecidine, a revolutionary accruement in impediment of the pathogen growth was determined with accruement in the concentration (Table 5). Comparable observations have been made with recognization to impact on weight of mycelium (Table 6). Antifungal activity of this product has been validated in earlier research. The antifungal activity exhibition might be because of the presence of organic acids (propanic, butyric, malic, succinic, and tartaric) (Hirose et al. 2000). Bajan et al. (1998) also observed a reduction in the vegetative growth of *Beauvaria bassiana* colonies caused by the industrial neem product. Different effects imply that neem oil protected the seeds of chickpea against the fungal sicknesses due to *Rhizoctonia solani*, *Sclerotium rolfsii* and *Sclerotim* (NRC 1992).

Saaf is also antifungal and was seen to impede the growth of the pathogen in all the concentrations and for the duration of the 30 days of the experiment (desk five). Its antifungal impacts are attributed to mancozeb and carbendazim which are the active elements. Results from a previous study by Baby and Mouli (2000), show that among fungicides screened, carbendazim was discovered to be the best in controlling thorny blight sickness of tea.

The usual fungicide, Topsin, inhibited growth at all the concentrations (desk 5). This confirms what other researchers said. Pathan et al. (2005) tested six fungicides against *Botryodiplodia theobromae*, the causal agent for mango gummosis. Topsin and Ridoml Gold have been found to be the most powerful in controlling the ailment beneath laboratory and field conditions. Antifungal activity of bark extracts of *W. ugandensis* was akin to that of control fungicides, Saaf and Topsin. They all inhibited the growth of *P. theae* in all concentrations and both in stable and liquid media (Tables 5 and 6). The neem product, Nimbecidine, was not as powerful as the standard fungicides and bark extracts, but it additionally inhibited a few percentages.

From the study, the neem product Nimbecidine was found to be more effective than Trilogy in impeding the accruement of *P. theae*. Bark extracts were the most effective among *W. ugandensis* extracts. The antifungal activity of the bark extracts became measurable to that of the studied fungicides (Saaf and Topsin). Root extracts were additionally energetic, but concentration pendent. They were most impeding at high concentration. The results from this study indicate that the plant products namely the neem products (especially Nimbecidine) and bark extracts from *W. ugandensis* substantially inhibited the growth of *P. theae*. Consequently, they have the capability to substitute or supplement fungicides to be used to control *P. theae* and consequently the branch and collar canker of tea resulted from the pathogen.

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Effects of Kenyan black tea water soluble components on theaflavins interaction with antibiotics against selected pathogenic bacteria

ANDREW BOR[1], EUCHARIA KENYA[2,♥], JOHN J.N. MBITHI[3], CHARLES MUTAI[4]

[1] School of Pure and Applied Sciences, Kentatta University, Nairobi, Kenya
[2]Department of Biochemistry and Biotechnology, Kenyatta University. Nairobi, Kenya. ♥email: kenya.eucharia@embuni.ac.ke
[3]Department of Medical Laboratory Science, Kenyatta University. Nairobi, Kenya
[4]Center for Traditional Medicine and Drug Research, Kenya Medical Research Institute. Nairobi, Kenya

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Abstract. *Bor A, Kenya E, Mbithi JJN, Mutai C. 2017. Effects of Kenyan black tea water soluble components on theaflavins interaction with antibiotics against selected pathogenic bacteria. Bioteknologi 14: 47-59.* This research is intended at differentiating the antibacterial activities of hot water extract of black tea having 18 µg/mL of theaflavins and 18 µg/mL of isolated theaflavins and their blend with antibiotics such as ampicillin. Their blended impacts with antibiotics were resolved by utilizing disk diffusion and modified Checkerboard method. The chi-square test was used to test the null hypothesis, which stated that water soluble components have no effect on theaflavins interaction with antibiotics. The water-soluble components of black tea extract were extracted with hot water, with the theaflavins being measured using the Flavognost method. Similar amounts of theaflavins were extracted using organic solvents and silica gel column chromatography. The concentrates of hot water extract and isolated theaflavins showed synergistic activity with selected antibiotics. However, the level of synergism differed significantly at $P<0.05$, with isolated theaflavins having a higher level. The difference in inhibitory effect between blended concentrates of hot water extract and isolated theaflavins with MIC (10.4 µg/mL) of ampicillin against *Salmonella typhi* was significant ($\chi^2 = 0.56$; $P<0.05$). The differences in inhibitory effect were also significant at ($\chi^2 = 0.699$; $P<0.05$) between the two black tea extracts blends with MIC (4.3 µg/mL) of norfloxacin against *Pseudomonas aeruginosa*. The blend of concentrates of hot water extract and isolated theaflavins with MIC (2 µg/mL) of ciprofloxacin differed significantly in level of impediment at ($\chi^2 = 1.98$; $P<0.05$) against *Staphylococcus aureus*. When the concentrates of the two black tea extracts were blended with MIC (5.25 µg/mL) of tetracycline, the inhibitory effect differed significantly at ($\chi^2 = 2.27$; $P<0.05$) against *Enterobacter aeruginosa*. It was also significant at ($\chi^2 = 0.4$; $P<0.05$) when concentrates of the two black tea extracts were blended with MIC (12 µg/mL) of chloramphenicol against *Escherichia coli*. The differences in inhibitory effect were attributed to the interactions within the tea infusion between water soluble components and theaflavins. Theaflavins in black tea infusions are being partially counteracted by one or more chemical components in it and it lowers the overall activity. However, the pattern of activity of isolated theaflavins and hot water extract of black tea were alike. This suggests that the theaflavins are the principal bioactive compounds in black tea infusions, despite the existence of interaction. Isolated theaflavins and hot water extracts of black tea restored the activity of lower concentrations of antibiotics below MIC to vulnerable breakpoints. The two black tea extracts together with antibiotics can be used in the treatment and prevention of bacterial infections.

Keywords: Antibiotics, Kenyan black tea, pathogenic bacteria, theaflavins, water soluble components

INTRODUCTION

Diseases caused by microbes that have become resistant to antibiotic drug therapy are an increasing public health problem. While the development of resistant strains is inevitable, the slack ways of administering and using antibiotics in human, veterinary medicine and in agriculture has greatly exacerbated the process (Kenneth 2008). Wound infections, gonorrhea, tuberculosis, pneumonia, septicemia, childhood ear infections and staphylococcal infections are just a few of the diseases that have become hard to treat with antibiotics (Amy 2008; Kenneth 2008).

Traditional methods of antibiotic discovery have failed to keep pace with the evolution of bacterial resistance, which suggests that new strategies to combat bacterial infections may be required (Peter and Floyd 2007). Microbial development of resistance, as well as economic incentives, have resulted in research and development in the search for new antibiotics to always maintain a pool of effective drugs (Kenneth 2008). Current research has focused on strengthening the antibacterial action of the existing antibiotics through blended therapy (Esimone et al. 2003; Nwafor et al. 2003). The advantages of blended antibiotic therapy are broadened spectrums of antimicrobial activity, occurrence of synergistic activity and prevention of bacterial resistance development (Aurer and Planeak 2004).

Sometimes, antibiotics are willfully or inadvertently consumed along with herbs or beverages. This shows the potential interaction between drugs and herbs, which can be beneficial or harmful. One of the herbs that are widely consumed concomitantly with most drugs is tea (Esimone et al. 2003). Research on tea has shown that it has some medicinal properties, including antimicrobial effect against a wide range of bacteria, fungi, and viruses (Sakanata et al. 1989; Toda et al. 1991).

The tea plant is member of *Camellia sinensis*. The two main varieties are *Camellia sinensis var sinensis* and

Camellia sinensis var. assamica. Tea is an infusion of the leaves of *Camellia sinensis* plant and is one of the most widely consumed beverages in the world (Higdon 2007). Tea is composed of several bioactive chemicals. The existence of alkaloids, saponins, tannins, catechin and other polyphenols in tea is revealed by the phytochemical screening. Recent research mainly focused on the potential health benefits of a class of compounds in tea known as flavonoids (Lai et al. 2001).

Flavonoids in fresh tea leaves are catechins which are a group of natural polyphenols (Lai et al. 2001). Another group of polyphenol pigments are theaflavins which are in black tea. Theaflavins are structured from polymerization of catechins due to oxidation by polyphenol oxidase at the fermentation stage during the manufacture of black tea. Theaflavins contribute to the characteristic bright orange-red color of black tea, accounting for approximate 2g/100g of the dried water extract of black tea (Higdon 2007). Catechins and theaflavins are believed to have a wide range of pharmaceutical benefits such as antibacterial, antihypertensive, antioxidative, hypolipidemic, antiviral and antifungal properties (Hara et al. 1991).

Current research on Japanese Sencha tea (green tea) and Indian Lipton brand black tea have shown that tea extracts have had effect on antibiotics effectiveness. Most of the research has been carried out on green tea extracts, rather than black tea extracts. Research using Indian Lipton brand black tea extracts showed synergistic activity with chloramphenicol and other antibiotics like gentamycin, methicillin and nalidixic acid against *Salmonella typhi*, *Shigella dysenteriae*, *Yersinia enterocolitica* and *Escherichia coli* (Tiwari et al. 2005). Gallic acid extract from black tea showed a synergistic effect with amikacin and sulfamethoxazole tested in a dose-dependent manner against *E. coli* (Tirang et al. 2007).

Research was done using hot water extract of Sencha (Japanese Green Tea) and methicillin to find out the blend effect. It was found that the extract of Sencha tea is not only capable of impeding methicillin resistant *Staphylococcus aureus* (MRSA), but also reestablishing the activity of methicillin against MRSA. Hara et al. (1991) also examined that the extract of tea acts synergistically with methicillin against MRSA. The synergistic effect was attributed to cathecins. The Sencha group of teas have high levels of ascorbic acid (Vitamin C), tannin and most of the catechins (Goto et al. 1996). Other research has indicated that Kenyan black teas have remarkable levels of the unoxidized flavan-3-ols (theaflavins and thearubigins) associated with human health (Owuor and Obanda, 1995).

The objectives of this research was (i) To determine antibacterial activities of hot water extract of Kenyan black tea on selected clinical isolates and standard bacteria (ii) To determine antibacterial activities of theaflavins in Kenyan black tea on selected clinical isolates and standard bacteria; (iii) To determine the effect of hot water extract of Kenyan black tea on the efficacy of blended theaflavins with selected antibiotics. (iv) To determine synergism between theaflavins and common antibiotics.

MATERIALS AND METHODS

Collection of sample materials

The commercial black tea which was processed and packed by Kenya Tea Packers Limited (KETEPA) was used as sample material and it was purchased in Nairobi

Preparation of hot water extract of black tea

The method of Yam et al. (1998) as described by Mbata et al. (2006) was used to extract the black tea with the aid of hot water. Into 100 mL of boiling water, two grams of tea was poured and left for 12 minutes and then it was sifted to make solution containing 2g/100 mL. The extract was freeze dried to powder form and stored at-4^0 C in refrigerator until it was needed.

Measurement of total theaflavins content of black tea

The Flavognost method (Hilton, 1973) was used to measure the total theaflavins in hot water. Briefly, 175 mL of boiling water was used to infuse black tea (9g). The infusion temperature was kept close to boiling point for 10 minutes. A vacuum flask with continuous mechanical shaking was used to carry out the infusion. When it was finished, that hot liquor was sifted and quickly cooled in cold water. Then, 10 mL aliquot of the filtered infusion was shaken for 10 minutes with the addition of 10 mL of isobutyl methyl ketone and, at last, the two layers were allowed to separate. Next, a mechanical shaker mixed 2 mL of the aliquot of the upper layer, 4 mL of ethanol and 2 mL of flavognost reagent (2% w/v diphenylboric acid-2-aminoethyl ester in ethanol) well. The mixture was left for 15 minutes at room temperature and the absorbance was read in spectrophotometer at 625nm. A mixture of isobutyl methyl ketone and ethanol (1:1, v/v) was used as blank. The content of theaflavins in black tea was calculated with the following formula:

$$\text{Theaflavins } (\mu \text{ mol/g}) = E_{625} \times 47{,}900/\text{ DM}$$

Where E_{625} was optical density, 47,900 was a constant and DM was the dried material of tea sample.

Extraction of theaflavins

Lai et al. (2001) method was used to extract theaflavins. 250g of black tea was weighed and extracted three times using 1.875l of 70% ethanol and then sifted. After the removal of ethanol in a rotary evaporator, the remaining water solution was extracted subsequently using chloroform (0.375l), ethyl acetate (0.25l) and butanol (0.25l). The ethyl acetate extract was implemented onto a silica gel column 80 (0.80 to 1.65cm i.d; silica gel 60M, 230-240 mesh). The total TF fraction was obtained when the column was purified with a mixture of chloroform and ethyl acetate 1:1 (v/v) followed by increasing the ratio of chloroform to ethyl acetate to 4:1 (v/v). The total theaflavin fraction was stored at -4^0C.

The purified fractions from the silica gel column were spotted on chromatographic plates with a thin layer of silica gel. They were then subjected to a mobile phase (solvent mixture) having ethyl acetate-acetic acid-water at

a ratio of 10:2:3. After being sprayed, the dissimilar compounds showed up as distinct spots at a distance from where they were spotted on the plates. The spots (compounds) that have the same relative mobility front (Rf) were merged. The absorbance of the fractions was measured at 380 and 460 nm using spectrophotometer. Theaflavins are known to have maximum absorbance at 380 nm which is especially related to their benzotropolone rings. The chemical (electron) shift around the benzotropolone ring that develops the bright red color of the theaflavins, is accountable for the absorbance maximum at 460 nm. The theaflavins fractions were confirmed by reacting 2 mL of theaflavins solution, 4 mL of ethanol and 2 mL of flavognost reagent (2% w/v diphenylboric acid-2-aminoethyl ester in ethanol). Diphenyl boric acid ethanolamine (Flavognost reagent) responded to the benzotropolone nucleus to form a green chromophore with a broad absorption maximum at 625 nm.

Preparation of tea extracts stock and working solutions

Two grams of black tea yielded 0.90 g when the water extract was freeze dried. Two-fold dilutions were produced to gain 100%, 50%, 25%, and 12.5% concentrations. 2 grams of the same black tea yielded 36µg or µmol of theaflavins. A doubling dilution of isolated theaflavins was produced to gain 100%, 50%, 25%, and 12.5% concentrations. The concentrations were stored at -4^{0}C until they were needed.

Preparation of antibiotic stock and working solutions

The antibiotics were taken off from storage (-20ºC) and allowed to come to room temperature. Each (250 mg) of the antibiotics were weighed and liquefied in suitable solvents and diluted in appropriate diluents (Appendix IV) to make a final 100 mL solution. The following formula was used to gain stock solutions:

$$W = \frac{1000 \times V\ (\text{mL}) \times C\ (\mu\text{g mL}^{-1})}{P\ (\mu\text{g mL}^{-1})}$$

Where:

P = potency given by manufacturer in relation to base C = final concentration of the solution

W = weight of antibiotic in mg to be liquefied in V = volume required in ml (20 mL)

The solutions supply of chloramphenicol, tetracycline, norfloxacin, ciprofloxacin and ampicillin were kept at-20 ^{0}C until they were needed.

Determination of MIC of antibiotics

Doubling dilutions of solution supply was produced to gain minimum inhibitory concentration (MIC) of each antibiotic against respective test organism using modified Bauer-Kirby method reported in the National Committee for Clinical Laboratory Standards (NCCLS 2002) report. Incubation was carried out at 37ºC for 24 hours and impediment zones were compared with those recommended by NCCLS (2002).

Preparation of blended concentrates of black tea extracts and antibiotics

A series of two-fold dilutions (1-⅛) were established using MIC of each antibiotic as the starting concentration. These concentrations were then blended andd merged with concentrations of hot water extract of black tea and isolated theaflavins (100%, 50%, 25% and 12.5%). Sterile discs were soaked in blended concentrations, air dried at room temperature and kept at-20ºC until they were needed.

Test organisms

Control and clinical isolates of *Escherichia coli, Enterobacter aeruginosa, Salmonella typhi, Pseudomonas aeruginosa, Staphylococcus aureus, E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 were from the National Public Health Laboratories, Nairobi, Kenya.

Identification of bacterial strains

Viability tests were done by picking the organisms from the stock using a sterile loop and inoculating into 9 mL of peptone water media. They were then incubated at 37ºC for 3 hours, followed by then sub-cultured in sterile agar plates having Mueller Hinton Agar (MHA) and incubated at 37ºC for 24 hours. Well isolated colonies were picked and used for identification.

Biochemical typing

Organisms from well separated colonies of akin appearance were taken from each of the plating media for further testing and an identification mark was put on the bottom of the Petri dish corresponding to the colonies. Five non-lactose fermenting (NLF) colonies were selected and put into separate tubes of Triple Sugar Iron agar (TSI), Simmon citrate and Urea agar. TSI was seeded by stabbing the butt and then streaking the slant with a zigzag configuration. Simmon citrate was also seeded by streaking the surface of the slant with a zigzag configuration. Urea agar was seeded by stabbing into medium four times. The test tubes with loose caps were incubated at 37ºC for 24 hours. The biochemical reactions were deciphered according to biochemical reaction chart for *Enterobacteriaceae, Aeromonas* and *Plesiomonas.*

Preparation of inoculants

One colony of each bacterial culture was selected and seeded into nutrient broth and incubated at 37ºC for 24 hours. The density of a bacterial suspension in nutrient broth was adjusted to match the turbidity of the 0.5 McFarland standard which was equivalent to 1.5 x 10^5 colony forming units (CFU)/mL.

Sensitivity test

An inoculum having 1.5 x 10^5 CFU/mL was taken off using a sterile cotton wrap and spread on Mueller Hinton agar plates. Impregnated discs were aseptically placed evenly on the surface of agar and pressed firmly. The plates were left for 3 hours to allow the antibiotics to diffuse. The inoculated plates were incubated overnight at 35ºC. The zones of impediment were measured using a transparent

ruler on the following morning after 18 hours incubation.

Chi-square test

The chi-square test was used to test the null hypothesis.

The formula for calculating chi-square (χ^2) is: $(\chi^2) = (O-E)^2/E$

Where: O is the examined result (mean impediment zone diameter of isolated theaflavins), and E is the expected result (mean impediment zone diameter of hot water extract of black tea).

RESULTS AND DISCUSSION

Comparison of inhibitory effect of isolated theaflavins and hot water extract of Kenyan black tea on *Salmonella typhi*

The inhibitory effect of hot water extract of black tea and isolated theaflavins are shown in Table 1. Isolated theaflavins showed stronger inhibitory effect as evidenced by large impediment zones. The 100%, 50%, and 25% concentrates of the two tea extracts impeded *S. typhi*. However, it resisted the 12.5% concentration of both extracts. Both 100% and 50% concentrations showed stronger inhibitory effect than minimum inhibitory concentration of ampicillin (10.4 µg/mL).

There was a statistically remarkable difference in the inhibitory effect between isolated theaflavins and hot water extract of black tea based on the diameters of zones of impediment at (χ^2 = 0.94; P<0.05). However, there was comparability in pattern of activity as the inhibitory effect increased, with increasing concentration of both tea extracts.

Comparison of inhibitory effect of hot water extract and isolated theaflavins of Kenyan black tea on *Pseudomonas aeruginosa*

Hot water extract of black tea showed lower inhibitory effect (smaller impediment zone diameter) on *P. aeruginosa* as compared to isolated theaflavins. Both 100% and 50% of the hot water extract of tea impeded *P. aeruginosa*, while 25% and 12.5% concentrates did not (Table 2). Only 12.5% concentrate of isolated theaflavins failed to inhibit *P. aeruginosa*. The 100%, 50% and 25% of isolated theaflavins and 100% and 50% of hot water extract showed stronger inhibitory effect than minimum inhibitory concentration of norfloxacin (6.4 µg/mL). The difference in inhibitory effect between isolated theaflavins and hot water extract of black tea was significant at (χ^2 = 1.02; P<0.05). There was comparability in pattern of activity.

Comparison of inhibitory effect of hot water extract and isolated theaflavins of Kenyan black tea on *Pseudomonas aeruginosa* standard (ATCC 27853)

All the concentrates of both hot water extract and isolated theaflavins, except the 12.5% dilution, impeded *P. aeruginosa* standard (ATCC 27853) (Table 3). Isolated theaflavins had a stronger impediment than hot water extract of black tea as examined in larger impediment zones. The 100%, 50% of both hot water extract and isolated theaflavins and 25% concentrates of isolated theaflavins also showed stronger inhibitory effect than minimum inhibitory concentration of norfloxacin (4.3 µg/mL).

There was a significant difference between inhibitory effect of concentrates of hot water extract and isolated theaflavins of black tea at (χ^2 = 0.56; P<0.05). The pattern of inhibitory effect was however akin. *P. aeruginosa* standard (ATCC 27853) was more susceptible to both tea extracts (Table 3) than *P. aeruginosa* (Table 2). *P. aeruginosa* resistance to hot water extract of black tea was high, showing a high difference in inhibitory effect with isolated theaflavins at (χ^2 = 1.02; P<0.05). The difference in inhibitory effect between the tea extracts on *P. aeruginosa* standard (ATCC 27853) was significantly lower at (χ^2 = 0.56; P<0.05).

Comparison of inhibitory effect on hot water extract and isolated theaflavins of Kenyan black tea on *Staphylococcus aureus* standard (ATCC 25923)

Isolated theaflavins effectively impeded *S. aureus* standard (ATCC 25923), as compared to hot water extract of black tea, as shown by larger impediment zone (Table 4). All the concentrates 100%, 50%, 25% and 12.5% of isolated theaflavins had stronger activity than minimum inhibitory concentration (1.2 µg/mL) of ciprofloxacin.

Only 12.5% concentration of hot water extract of black tea had a lower activity than the minimum inhibitory concentration (1.2 µg/mL) of ciprofloxacin. There was a statistical difference in inhibitory effect between isolated theaflavins and hot water extract of black tea on *S. aureus* standard (ATCC 25923) at (χ^2 = 4.42; P<0.05).

Comparison of inhibitory effect of hot water extract and isolated theaflavins of Kenyan black tea on *Staphylococcus aureus*

Hot water extract of black tea had lower inhibitory activity on *S. aureus* than isolated theaflavins (Table 5). Isolated theaflavins effectively impeded with large zones of impediment. All the concentrates 100%, 50%, 25% and 12.5% of isolated theaflavins showed stronger inhibitory effect than the minimum inhibitory concentration (2 µg/mL) of ciprofloxacin. Only 12.5% concentrate of hot water extract showed lower inhibitory effect than minimum inhibitory concentration (2 µg/mL) of ciprofloxacin. The inhibitory effect between isolated theaflavins and hot water extract of black tea was significant at (χ^2 = 1.01; P<0.05).

Comparison of inhibitory effect of hot water extract and isolated theaflavins of Kenyan black tea on *Enterobacter aeruginosa*

The 12.5% concentrates of hot water extract and isolated theaflavins did not inhibit *E. aeruginosa*. However, isolated theaflavins showed stronger inhibitory effect than hot water extract of black tea (Table 6). Only 25% concentration of hot water extract of tea showed lower inhibitory effect than minimum inhibitory concentration of (5.25 µg/mL) of tetracycline. The 100%, 50% and 25% of

isolated theaflavins showed stronger inhibitory effect than minimum inhibitory concentration (5.25 µg/mL) of tetracycline. The difference in level of inhibitory effect between isolated theaflavins and hot water extract of black tea on *E. aeruginosa* was significant at ($\chi^2 = 3.04$; $P<0.05$).

Comparison of inhibitory effect of hot water extract and isolated theaflavins of Kenyan black tea on *Escherichia coli*

The 100%, 50%, 25% and 12.5% concentrations of both black tea extracts effectively impeded *E. coli*. However, isolated theaflavins showed stronger activity with larger zones of impediment (Table 7). All the concentrates of hot water extract and isolated theaflavins showed better inhibitory effect than the minimum inhibitory concentration of chloramphenicol. The difference in inhibitory effect between concentrates of isolated theaflavins and hot water extracts of black tea was significant at ($\chi^2 = 1.62$; $P<0.05$). The pattern of inhibitory effect was akin as it increased with increasing concentration of both tea extracts.

Comparison of inhibitory effect of hot water extract and isolated theaflavins of Kenyan black tea on *Escherichia coli* standard (ATCC 25922)

Escherichia coli standard (ATCC 25922) was effectively impeded by hot water extract and isolated theaflavins of black tea (Table 8). However, hot water extract of black tea showed lower inhibitory effect as shown by smaller zones of impediment than that of isolated theaflavins. Both tea extracts have a strong impediment to *E. coli* standard (ATCC 25922) than the minimum inhibitory concentration (8.4 µg/mL) of chloramphenicol. The inhibitory effect between concentrates of isolated theaflavins and hot water extracts was significant at ($\chi^2 = 1.74$; $P<0.05$). This difference was slightly higher than that of *E. coli* ($\chi^2 = 1.62$; $P<0.05$).

The effect of hot water extract of Kenyan black tea on the efficacy of antibiotics

The 100%, 50% and 25% concentrates of hot water extract of black tea with minimum inhibitory concentration (10.4 µg/mL) of ampicillin acted synergistically against *S. typhi* as shown in Figure 1.A. The examination of the sum of activity of concentrates of hot water extract of black tea with MIC of ampicillin as presented in Table 1 did not exceed that presented in Figure 1.A. The doubling dilutions of the MIC (5.2, 2.6 and 1.3 µg/mL) of ampicillin had no activities of their own. Their activities were restored when they were blended with 100%, 50% and 25% concentrates of hot water extract of black tea. Those were also seen as the activities of the blended hot water concentrates with 5.2, 2.6 and 1.3 µg/mL of ampicillin (Figure 1.A), which were exceeded that of the concentrates of tea extract only as presented in Table 1. The restoring of activity of ampicillin clearly demonstrated synergism with hot water extract of black tea.

Synergism between MIC (4.3 µg/mL) of norfloxacin, a fluoroquinolone and the 100% and 50% concentrates of hot water extract of black tea against *P. aeruginosa* was also examined (Figure 1.B). That compared with that of the sum of activity of individual concentrate of hot water extract of black tea and MIC of norfloxacin is presented in Table 2. The two concentrates of tea extract were also capable of restoring the activity of the doubling dilutions (2.15 and 1.075 µg/mL) of MIC of norfloxacin against *P. aeruginosa*.

The synergism of hot water extract of black tea with MIC of norfloxacin was further examined against *P. aeruginosa* (ATCC 27853). The bacteria was less resistant to the hot water extract of black tea than *P. aeruginosa*, resisting only the 12.5% concentrate (Figure 1.C). Therefore, the impediment zones of blended concentrates of hot water extract with MIC of norfloxacin against *P. aeruginosa* (ATCC 27853) were larger than those in *P. aeruginosa* cultures. This difference was also examined in activity of blended hot water extract of black tea with doubling dilutions of MIC of norfloxacin. The activities of doubling dilutions of MIC of norfloxacin were also restored.

Synergistic effect of hot water extract of black tea with fluoroquinolone was also examined with MIC of ciprofloxacin against *S. aureus*. The *S. aureus* was very susceptible to the blended concentrates (Figure 1.D). The hot water extract of black tea also restored the activity of doubling dilutions of MIC of ciprofloxacin (1, 0.5 and 0.25 µg/mL).

Synergism of hot water extract of black tea with MIC of ciprofloxacin was also examined when *S. aureus* (ATCC 25923) was used as test bacteria (Figure 1.E). The susceptibility of *S. aureus* (ATCC 25923) to blended concentrates of hot water extracts with MIC and its doubling dilutions of ciprofloxacin was high as compared to that of *S. aureus* (Figure 1.D).

Tetracycline with hot water extract of black tea acted synergistically against *E. aeruginosa*. Synergism was examined when 100%, 50% and 25% concentrates of hot water extract of black tea were blended with MIC (5.25 µg/mL) of tetracycline. Hot water extract was also capable of restoring the activity of doubling dilutions (2.625, 1.3125 and 0.656 µg/mL) of tetracycline (Figure 1.F).

The blend of MIC (12 µg/mL) of chloramphenicol with 100%, 50%, 25% and 12.5% concentrates of hot water extract of black tea also showed synergistic effect against *E. coli* (Figure 1.G). *E. coli* was very susceptible to the blended concentrates of hot water extract of black tea with MIC of chloramphenicol. This was examined in large impediment zones around the discs having these blends. The concentrates of hot water extract of black tea restored the activity of doubling dilutions (6, 3 and 1.5 µg/mL) of MIC of chloramphenicol.

Synergism of MIC of chloramphenicol with concentrates of hot water extract of black tea was also examined when *E. coli* (ATCC 25922) was used as test bacteria (Figure 1.H). The MIC of chloramphenicol against *E. coli* (ATCC 25922) was 8.4 µg/mL compared to 12 µg/mL of *E. coli*. *E. coli* (ATCC 25922) was therefore more susceptible to the blended MIC of chloramphenicol with concentrates of hot water extract of black tea as examined in large impediment zones (Figure 1.H). That was also examined in blended activity of doubling dilutions and 1.05 µg/mL) of MIC (8.4 µg/mL) of chloramphenicol (Figure 1.H).

Table 1. Antibacterial activity of hot water extract and isolated theaflavins of Kenyan black tea and MIC of ampicillin against Salmonella typhi.

Concentrates	N	Mean impediment zone diameter in mm		
		Ampicillin	Hot water extract	Isolated theaflavins
100%	3		14.3	16
50%	3		10.5	13.4
25%	3		7.2	8.9
12.5%	3		NI	NI
MIC of amp (10.4 µg/mL)		9.4		

Note: NI: no impediment, amp: ampicillin, N: number of replicates

Table 2. Antibacterial activity of hot water extract and isolated theaflavins of Kenyan black tea and MIC of norfloxacin against *Pseudomonas aeruginosa.*

Concentrates	N	Mean impediment zone diameter in mm		
		Norfloxacin	Hot water extract	Isolated theaflavins
100%	3		11	13
50%	3		8.34	10.7
25%	3		NI	9.2
12.5%	3		NI	NI
MIC of norf (6.4 µg/mL)		6.89		

Note: NI: no impediment, norf: norfloxacin, N: number of replicates

Table 3. Antibacterial activities of varying concentrations of isolated theaflavins and hot water extract of Kenyan black tea and MIC of norfloxacin against *Pseudomonas aeruginosa* standard (ATCC 27853).

Concentrates	N	Mean impediment zone diameter in mm		
		Norfloxacin	Hot water extract	Isolated theaflavins
100%	3		14	15.68
50%	3		11.96	13.59
25%	3		8.3	9.4
12.5%	3		NI	NI
MIC of norf (4.3 µg/mL)		8.5		

Note: NI: no impediment, norf: norfloxacin, N: number of replicates

Table 4. Antibacterial activity of varying concentrations of isolated theaflavins and hot water extract of Kenyan black tea and MIC of ciprofloxacin against *Staphylococcus aureus* (ATCC 25923).

Concentrates	N	Mean impediment zone diameter in mm		
		Ciprofloxacin	Hot water extract	Isolated theaflavins
100%	3		17	20
50%	3		15.4	17.96
25%	3		12.9	14.5
12.5%	3		10	12.2
MIC of Cipro (1.2 µg/mL)		11		

Note: Cipro: ciprofloxacin, N: number of replicates

Table 5. Antibacterial activities of varying concentrations of isolated theaflavins and hot water extract of Kenyan black tea and MIC of ciprofloxacin against *Staphylococcus aureus*.

Concentrates	N	Mean impediment zone diameter in mm		
		Ciproflox acin	Hot water extract	Isolated theaflavins
100%	3		15	19
50%	3		13.7	16.8
25%	3		11.8	14.15
12.5%	3		9.2	12.6
MIC of Cipro (2 µg/mL)		9.8		

Note: Cipro: ciprofloxacin, N: number of replicates

Table 6. Antibacterial activities of varying concentrations of isolated theaflavins and hot water extract of Kenyan black tea and MIC of tetracycline against *Enterobacter aeruginosa.*

Concentrates	N	Mean impediment zone diameter in mm		
		Tetracycline	Hot water extract	Isolated theaflavins
100%	3		14.2	17
50%	3		11	14
25%	3		6.9	10.3
12.5%	3		NI	NI
MIC of tet (5.25 µg/mL)		7.8		

Note: NI: no impediment, tet: tetracycline, N: number of replicates

Table 7. Antibacterial activities of varying concentrations of isolated theaflavins hot water extract of Kenyan black tea and MIC of chloramphenicol against *Escherichia coli.*

Concentrates	N	Mean impediment zone diameter in mm		
		Chloramphenicol	Hot water extract	Isolated theaflavins
100%	3		13	16
50%	3		11.6	14.4
25%	3		9.8	10.56
12.5%	3		7.2	8.4
MIC of chlo (12 µg/mL)		6.8		

Note: Chlo: chloramphenicol, N: number of replicates

Table 8. Antibacterial activities of varying concentrations of isolated theaflavins and hot water extract of Kenyan black tea and MIC of chloramphenicol against *Escherichia coli* standard (ATCC 25922).

Concentrates	N	Mean impediment zone diameter in mm	
100%	3	15	18
50%	3	12.8	15.2
25%	3	10.4	12.56
12.5%	3	8.35	9.4
MIC of tet (8.4 µg/mL)		8	

Note: Chlo: chloramphenicol, N: number of replicates

Synergism of isolated theaflavins of Kenyan black tea with antibiotics

Synergistic antibacterial activities of isolated theaflavins and ampicillin

A synergistic antibacterial activity of isolated theaflavins of black tea and ampicillin against *S. typhi* is presented in Figure 2.A. Isolated theaflavins acted synergistically with minimum inhibitory concentration (10.4 µg/mL) of ampicillin. Isolated theaflavins like hot water extract of black tea, restored the activity of doubling dilutions (5.2, 2.6 and 1.3 µg/mL) of MIC of ampicillin. Otherwise, the blended activity of isolated theaflavins and doubling dilutions of MIC of ampicillin would have been equal to that of concentrates of isolated theaflavins alone (Table 1) if activity was not restored.

Synergistic antibacterial activity of isolated theaflavins and norfloxacin

Isolated theaflavins showed synergistic activity with norfloxacin, a fluoroquinolone against *P. aeruginosa* (Figure 2.B). Synergism was examined when 100%, 50% and 25% concentrates of isolated theaflavins were blended with MIC (6.4 µg/mL) of norfloxacin. The concentrates of isolated theaflavins also restored the activity of doubling dilutions (2.5 and 1.075 µg/mL) of norfloxacin.

The synergistic antibacterial activity of isolated theaflavins and norfloxacin was also examined when *P. aeruginosa* (ATCC 27853) was used instead of *P. aeruginosa*. However, the level of synergism and restored activity differed. *P. aeruginosa* (ATCC 27853) was less resistant (Figure 2.C) with the MIC (4.3 µg/mL) of norfloxacin against it.

Synergistic antibacterial activity of isolated theaflavins and ciprofloxacin

The synergistic effects between 100%, 50%, 25% and 12.5% concentrates of isolated theaflavins and MIC (2 µg/mL) of ciprofloxacin against *S. aureus* was examined (Figure 2.D).These blends effectively impeded *S. aureus* as shown by large impediment zones in the cultures. Isolated theaflavins also restored the activity of doubling dilutions (1, 0.5 and 0.25 µg/mL) of ciprofloxacin.

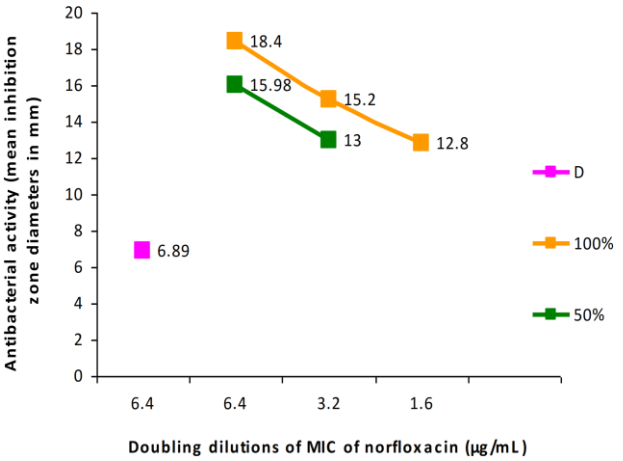

Figure 1.B. Antibacterial activities of blended concentrates of hot water extracts of black tea with doubling dilutions of MIC (6.4 µg/mL) of norfloxacin against *Pseudomonas aeruginosa*. D-Impediment zone diameter of minimum inhibitory concentration (6.4 µg/mL) of norfloxacin, 100%-undiluted hot water extract, 50%-a half dilution of 100% concentrate, N = 3.

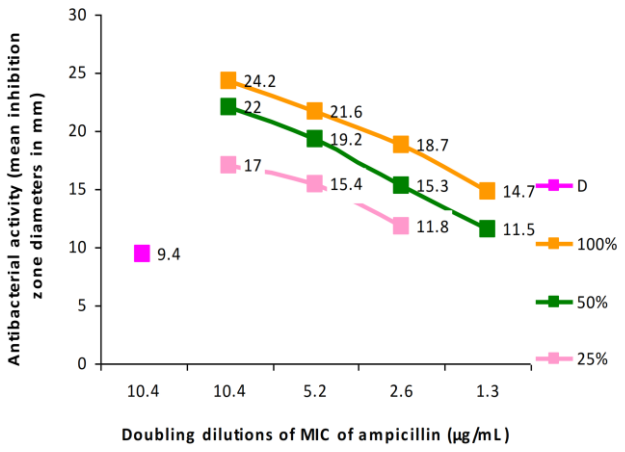

Figure 1.A. Antibacterial activities of blended concentrates of hot water extracts of black tea with doubling dilutions of MIC of ampicillin against *Salmonella typhi*. D-Impediment zone diameter of minimum inhibitory concentration (10.4 µg/mL) of ampicillin, 100%-undiluted hot water extract, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, 12.5%-dilution of 100% to ⅛ of its original concentration, N = 3.

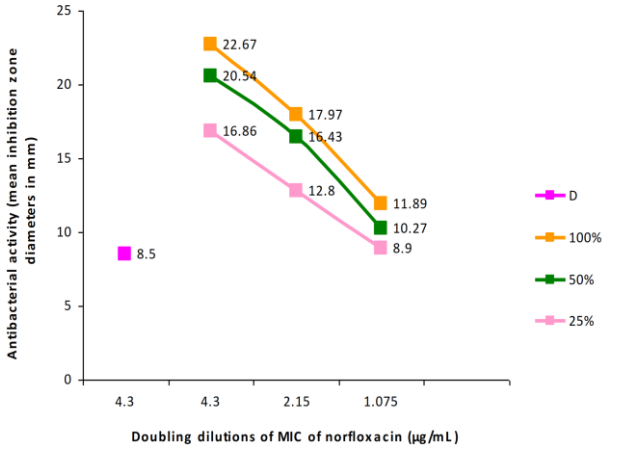

Figure 1.C. Antibacterial activities of blended concentrates of hot water extracts of black tea with doubling dilutions of MIC (4.3 µg/mL) of norfloxacin against *Pseudomonas aeruginosa (*ATCC 27853*)*. D-Impediment zone diameter of minimum inhibitory concentration (4.3 µg/mL) of norfloxacin, 100%-undiluted hot water extract, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, N = 3.

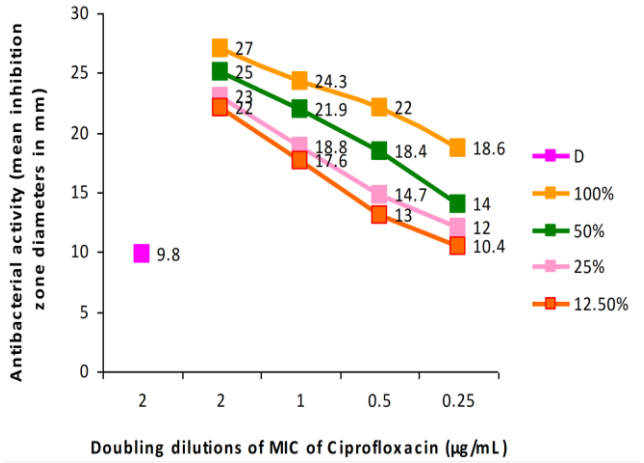

Figure 1.D. Antibacterial activities of blended concentrates of hot water extracts of black tea with doubling dilutions of MIC (2 µg/mL) of ciprofloxacin against *Staphylococcus aureus*. D-Impediment zone diameter of minimum inhibitory concentration (2 µg/mL) of ciprofloxacin, 100%-undiluted hot water extract, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, 12.5%-dilution of 100% to ⅛ of its original concentration, N = 3.

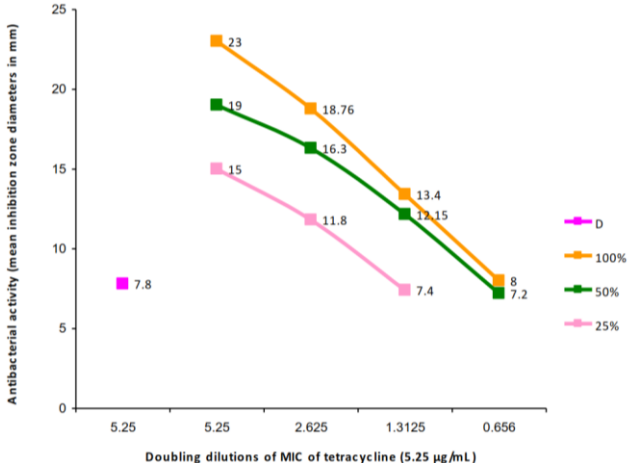

Figure 1.F. Antibacterial activities of blended concentrates of hot water extracts of black tea with doubling dilutions of MIC (5.25 µg/mL) of tetracycline against *Enterobacter aeruginosa*. D-Impediment zone diameter of minimum inhibitory concentration (5.25 µg/mL) of tetracycline, 100%-undiluted hot water extract, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, N = 3.

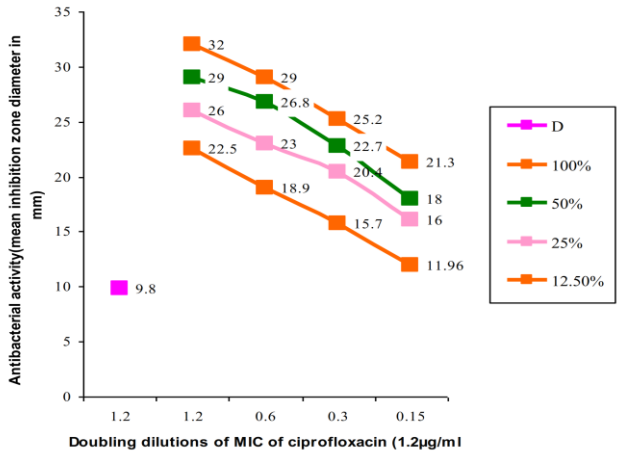

Figure 1.E. Antibacterial activities of blended concentrates of hot water extracts of black tea with doubling dilutions of MIC (1.2 µg/mL) of ciprofloxacin against *Staphylococcus aureus* (ATCC 25923). D-Impediment zone diameter of minimum inhibitory concentration (1.2 µg/mL) of ciprofloxacin, 100%-undiluted hot water extract, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, 12.5%-dilution of 100% to ⅛ of its original concentration, N = 3.

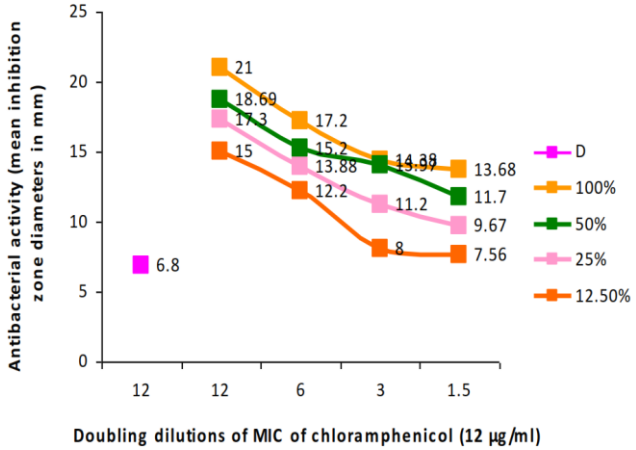

Figure 1.G. Antibacterial activity of blended concentrates of hot water extracts of black tea with doubling dilutions of MIC (12 µg/mL) of chloramphenicol against *Escherichia coli*. D-Impediment zone diameter of minimum inhibitory concentration (12 µg/mL) of chloramphenicol, 100%-undiluted hot water extract, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, N = 3.

The synergistic activity between MIC of ciprofloxacin and concentrates of isolated theaflavins was clearly demonstrated when *S. aureus* (ATCC 25923) was used instead of *S. aureus* (Figure 2.E). That was examined in larger impediment zones in *S. aureus* (ATCC 25923) cultures. Also, the activity of doubling dilutions (0.6, 0.3 and 0.15 µg/mL) of ciprofloxacin was restored by isolated theaflavins.

Synergistic antibacterial activity of isolated theaflavins and tetracycline

The blend of isolated theaflavins concentrates (100%, 50% and 25%) and minimum inhibitory concentration (5.25 µg/mL) of tetracycline showed synergistic effect. That was examined when activity was tested on *E. aeruginosa* (Figure 2.F). Isolated theaflavins concentrates restored the activity of tetracycline when blended with its doubling dilutions (2.625, 1.3125 and 0.656 µg/mL). *E. aeruginosa* has developed resistance to antibiotics. The blended formulation of tetracycline and isolated theaflavins effectively impeded.

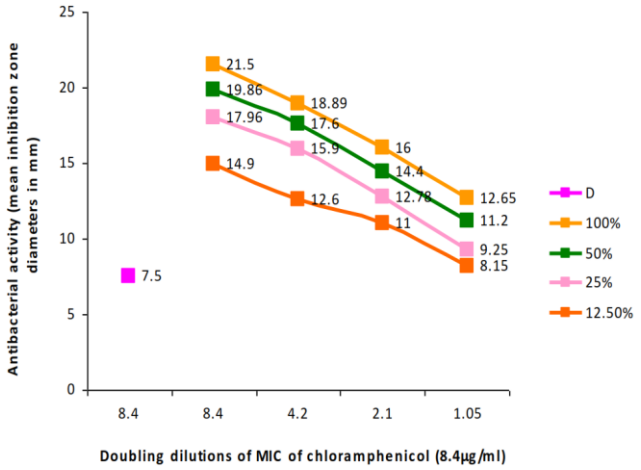

Figure 1.H. Antibacterial activities of blended concentrates of hot water extracts of black tea with doubling dilutions of MIC (8.4 µg/mL) of chloramphenicol against *Escherichia coli* (ATCC 25922). D-Impediment zone diameter of minimum inhibitory concentration (8.4 µg/mL) of chloramphenicol, 100%-undiluted hot water extract, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, N = 3.

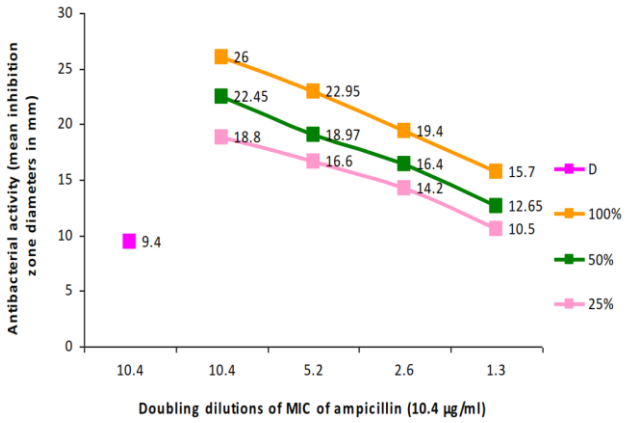

Figure 2.A. Antibacterial activities of blended concentrates of isolated theaflavins of black tea with doubling dilutions of MIC of ampicillin against *Salmonella typhi.* D-Impediment zone diameter of minimum inhibitory concentration (10.4 µg/mL) of ampicillin, 100%-undiluted isolated theaflavins, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, N = 3.

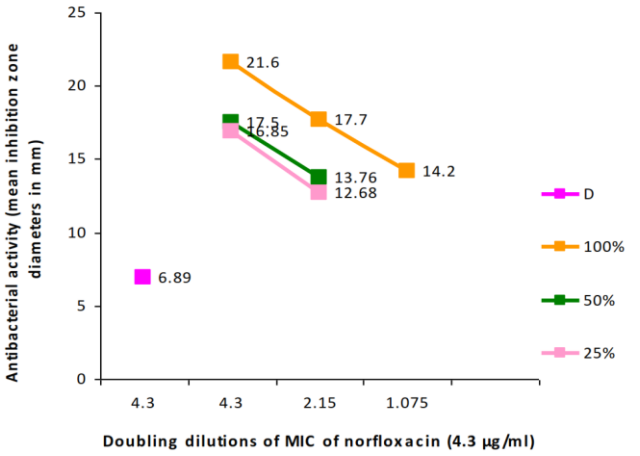

Figure 2.B. Antibacterial activities of blended concentrates of isolated theaflavins of black tea with doubling dilutions of MIC (4.3 µg/mL) of norfloxacin against *Pseudomonas aeruginosa*. D-Impediment zone diameter of minimum inhibitory concentration (4.3 µg/mL) of norfloxacin, 100%-undiluted isolated, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, N = 3.

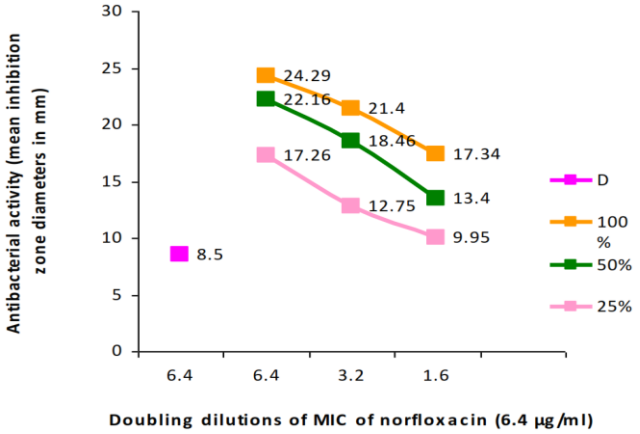

Figure 2.C. Antibacterial activities of blended concentrates of isolated theaflavins of black tea with doubling dilutions of MIC of norfloxacin against *Pseudomonas aeruginosa (*ATCC 27853*).* D-Impediment zone diameter of minimum inhibitory concentration (6.4 µg/mL) of norfloxacin, 100%-undiluted isolated theaflavins, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, N = 3.

Synergistic antibacterial activity of isolated theaflavins and chloramphenicol

Isolated theaflavins and chloramphenicol showed strong synergistic effect against *E. coli*. This was examined when minimum inhibitory concentration (12 µg/mL) of chloramphenicol was blended with 100%, 50%, 25% and 12.5% concentrates of isolated theaflavins (Figure 2.G). The blend effectively impeded *E. coli*, as shown by large impediment zones. The activity of chloramphenicol was also restored when doubling dilutions (6, 3 and 1.5 µg/mL) of its MIC were blended with isolated theaflavins concentrates. The doubling dilutions have no activity against *E. coli* on their own. The resulting activity after blend with isolated theaflavins concentrates exceeded that of the concentrates only. That indicated doubling dilutions of MIC contributed to the overall activity. Synergism was also examined when *E. coli* (ATCC 25922) was used as test bacteria (Figure 2.H).

The 100%, 50%, 25% and 12.5% concentrates of isolated theaflavins were blended with MIC (8.4 µg/mL) of chloramphenicol against *E. coli* (ATCC 25922). The differences in impediment in *E. coli* and *E. coli* (ATCC 25922) cultures was due to the differences in susceptibility.

The effect of other chemical components in black tea infusion on theaflavins interaction with antibiotics

Isolated theaflavins showed stronger inhibitory effect against the bacterial species tested. The differences in inhibitory effect was significant at (P<0.05). The difference in inhibitory effect between blended concentrates of hot water extract and isolated theaflavins with MIC (10.4 µg/mL) of ampicillin against *S. typhi* was significant at (χ^2 = 0.56; P<0.05). There was akin observation when dissimilar bacteria species and dissimilar antibiotics were used. The differences in inhibitory effect was significant at (χ^2 = 0.699; P<0.05) between the two black tea extracts blends with MIC (4.3 µg/mL) of norfloxacin against *P. aeruginosa*. It was also significant (χ^2 = 0.425; P<0.05) when *P. aeruginosa* (ATCC 27853) was used as test bacteria instead of *P. aeruginosa*. The blend of concentrates of hot water extract and isolated theaflavins with MIC (2 µg/mL) of ciprofloxacin differed significantly in level of impediment at (χ^2 = 1.98; P<0.05) against *S. aureus*. The difference in inhibitory effect was significant (χ^2 = 0.67; P<0.05) when *S. aureus* (ATCC 25923) was used instead. When the concentrates of the two black tea extracts were blended with MIC (5.25 µg/mL) of tetracycline, the inhibitory effect differed remarkably at (χ^2 = 2.27; P<0.05) against *E. aeruginosa*. Akin observation was also remarkable at (χ^2 = 0.4; P<0.05) when concentrates of the two black tea extracts were blended with MIC (12 µg/mL) of chloramphenicol against *E. coli*. While the difference in inhibitory effect was highly significant at (χ^2 = 1.039; P<0.05) when *E. coli* (ATCC 25922) was used instead.

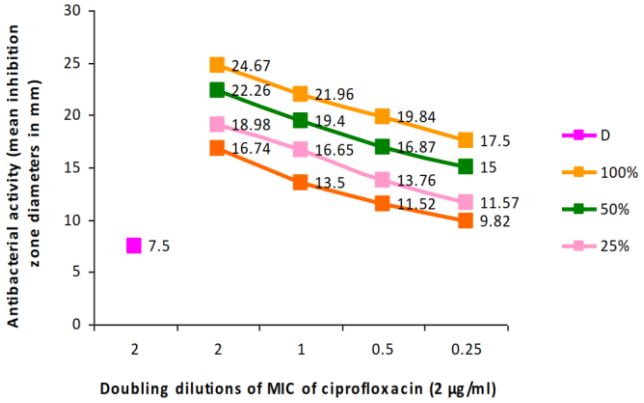

Figure 2.D. Antibacterial activities of blended concentrates of isolated theaflavins of black tea with doubling dilutions of MIC of ciprofloxacin against *Staphylococcus aureus.* D-Impediment zone diameter of minimum inhibitory concentration (2 µg/mL) of ciprofloxacin, 100%-undiluted hot water extract, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, 12.5%-dilution of 100% to ⅛ of its original concentration, N = 3.

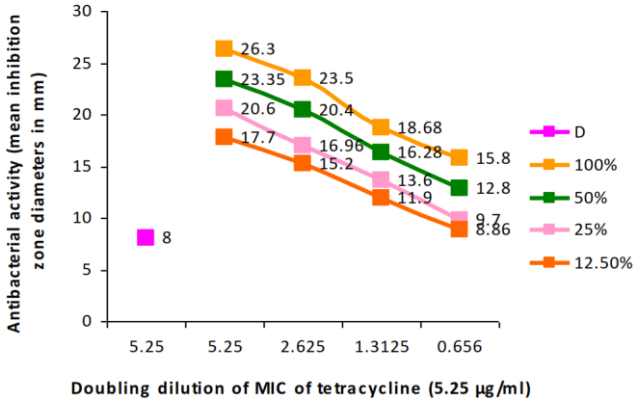

Figure 2.F. Antibacterial activities of blended concentrates of isolated theaflavins of black tea with doubling dilutions of MIC (5.25 µg/mL) of tetracycline against *Enterobacter aeruginosa.* D-Impediment zone diameter of minimum inhibitory concentration (5.25 µg/mL) of tetracycline, 100%-undiluted hot water extract, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, N = 3.

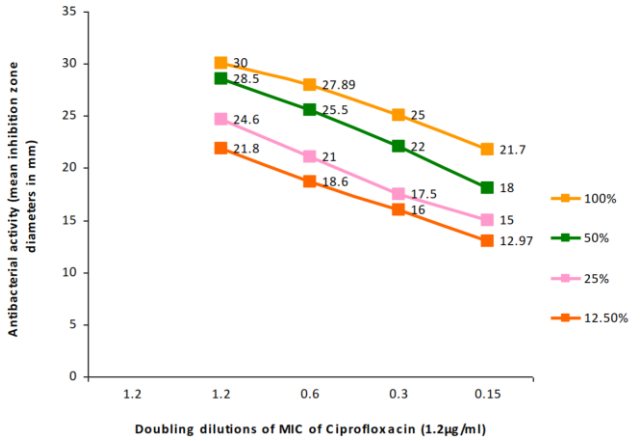

Figure 2.E. Antibacterial activities of blended concentrates of isolated theaflavins of black tea with doubling dilutions of MIC (1.2 µg/mL) of ciprofloxacin against *Staphylococcus aureus* (ATCC 25923). D-Impediment zone diameter of minimum inhibitory concentration (1.2 µg/mL) of ciprofloxacin, 100%-undiluted isolated theaflavins, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, 12.5%-dilution of 100% to ⅛ of its original concentration, N = 3.

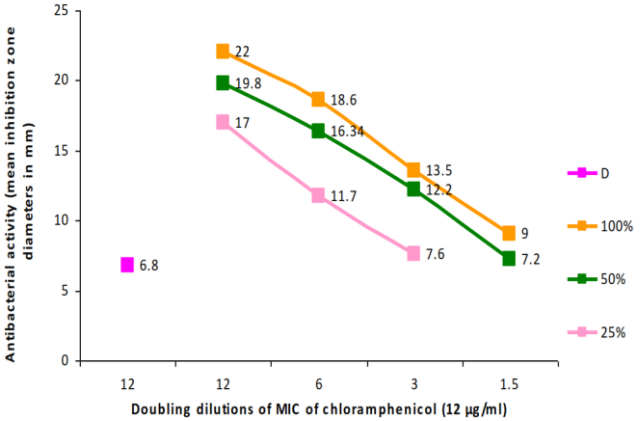

Figure 2.G. Antibacterial activities of blended concentrates of isolated theaflavins of black tea with doubling dilutions of MIC (12 µg/mL) of chloramphenicol against *Escherichia coli*. D-Impediment zone diameter of minimum inhibitory concentration (12 µg/mL) of chloramphenicol, 100%-undiluted isolated theaflavins, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, N = 3.

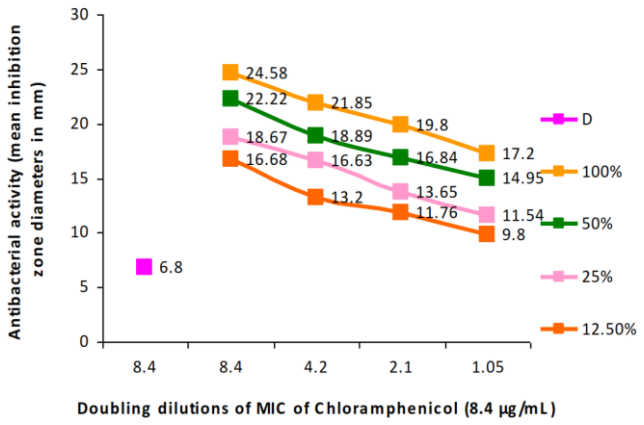

Figure 2.H. Antibacterial activities of blended concentrates of isolated theaflavins of black tea with doubling dilutions of MIC (8.4 µg/mL) of chloramphenicol against *Escherichia coli* (ATCC 25922). D-Impediment zone diameter of minimum inhibitory concentration (8.4 µg/mL) of chloramphenicol, 100%-undiluted isolated theaflavins, 50%-a half dilution of 100% concentrate, 25%-dilution of 100% to ¼ of its original concentration, N = 3.

Discussion

The present *in vitro* research clearly demonstrated that hot water extract of Kenyan black tea and theaflavins isolated from the same tea exhibit synergism with ampicillin, tetracycline, chloramphenicol, ciprofloxacin and norfloxacin. The synergistic activity was examined when minimum inhibitory concentration (MIC) of each antibiotic was blended with varying concentrations of isolated theaflavins and hot water extract of black tea. The two black tea extracts also restored the activity of lower concentrations (doubling dilutions of MIC) of antibiotics to susceptible breakpoints. The low concentrations of antibiotics had no activity of their own. The restoration of activity of antibiotics confirmed synergism between antibiotics and the tea extracts. The synergism examined in Kenyan black tea concurred with other research on dissimilar types of tea. However, most of the research has looked at green tea extracts, compared to black tea extracts.

Hot water extract of Indian Lipton brand black tea showed synergistic activity with chloramphenicol, gentamicin, methillicin and nalidixic acid against enteropathogens. Growth impediment of *S. dysenteriae* at low concentration of chloramphenicol (2.5 µg/mL) and tea extract (5.09 mg/mL) as compared to MIC of individual agent (chloramphenicol 5 µg/mL or black tea extract 9.09 mg/mL) further confirmed the synergistic activity (Tiwari et al. 2005).

Synergistic microbial growth impediment by Indian Lipton brand black tea extract and antibiotics was attributed to the presence of dual binding sites on the bacterial surface for antibiotic and tea extract. The results agreed with the marked reduction in MIC of oxacillin and other beta-lactams antibiotics as reported in presence of epicatechin gallate in methicillin-resistant *Staphylococcus aureus* (Tiwari et al. 2005).

The enhanced effect of Japanese green tea on inhibitory activities of antibiotics against MRSA strains have also been examined. The synergistic activity of hot water extract of Sencha (Japanese green tea) with methillicin against methicillin-resistant *Staphylococcus aureus* demonstrated possible benefits of tea extracts (Hara et al. 1991). The extract of Sencha tea was not only capable of inhibiting methillicin-resistant *Staphylococcus aureus*, but also restoring the activity of methillicin. The antibacterial activity of green tea can be explained by its content of (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG) and (-)-epicatechin-3-gallate (ECG) (Saroj et al. 1997).

The varying concentrations of hot water extract and isolated theaflavins of Kenyan black tea showed antibacterial activity against *E. coli*, *E. aeruginosa*, *S. typhi*, *P. aeruginosa*, *S. aureus*, *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 25923). This preliminary research of the antibacterial activities of hot water extract of processed Kenyan black tea and Nigerian Lipton tea showed inhibitory effect against *V. cholerea*, *E. coli*, *Salmonella* species, *P. aeruginosa*, *Proteus* species and *S. aureus*. The Kenyan tea showed more inhibitory actions on most of the organisms tested. The zones of impediment produced by Kenyan tea on test organisms were generally larger than those produced by the Nigerian Lipton tea. This could be because it contains more active ingredients (phytochemical substances) than the Nigerian tea, which resulted in a stronger inhibitory effect on the test organism (Mbata 2006).

Theaflavins have also been reported to have antibacterial activities against *T. mentagophytes*, *T. rubrum*, *C. albicans* and *Cryp. neoformans* (Okubo et al. 1991). Kenyan tea clones generally produce black tea with high levels of total theaflavins (Owuor and Obanda, 1995).

Hot water extract and isolated theaflavins of Kenyan black tea differed in strength of antibacterial activities. Akin difference was examined when the two black tea extracts were blended with antibiotics. The antibacterial activities and synergistic activity with antibiotics was lower for hot water extract, as compared to that of isolated theaflavins. That was even though hot water extract tested had the same amount 36 µmol (18 µmol /g) of theaflavins as isolated theaflavins (36 µmol/g). The differences in inhibitory effect examined were attributed to interactions within the tea infusion between water soluble components and theaflavins that were not isolated. Theaflavins in black tea infusion are being partially antagonized by one or more chemical components in it lowering the overall activity. The differences in inhibitory effect was significant ($P<0.05$). However, the pattern of activity of isolated theaflavins and hot water extract (infusion) of black tea were akin. This suggested that the theaflavins that were not isolated were the principal bioactive compounds in black infusion despite the existence of interactions. This inference agrees with research by Okubo et al. (1991) that showed theaflavins as the major antibacterial compound in tea. Further, Apostolides and Weisberger (1995) remarkably showed that theaflavins as the principal quality components in black tea are beneficial to human health. In addition to theaflavins, other antibacterial compounds in hot water extract of black tea are catechins, fluoride,

kaempferol, quercitin and myericitin (Higdon 2007). However, the blend of these in the hot water extract in the present research does not boost the antibacterial activity of theaflavins that were not isolated to exceed that of isolated theaflavins.

These compounds isolated from other plants have been found to have antibacterial properties. Kaempferol (3,4',5,7-tetrahydroxyflavone) and quercetin (3,3',4',5,7-pentahydroxyflavone) showed the lowest minimum inhibitory concentrations (MICs) against the clinical MRSA (Lin et al. 2008). Tryptanthrin and kaempferol isolated from the indigo plant (*Polygonum tinctorium lour*) remarkably decreased the numbers of *H. pylori* colonies in a dose-dependent manner (Maria et al. 2005). Querticin and kaempferol from other plants have been shown to have blended effects with antibiotics. Blends of rifampicin and either with kaempferol or quercetin acted synergistic ally or partially synergistic ally against the clinical MRSA. Rifampicin blended with kaempferol, or quercetin exhibited good beta-lactamase inhibitory effects (57.8 % and 75.8 %, respectively) against a representative isolate (Lin et al. 2008).

The development of bacterial resistance to antibiotics can be prevented by hot water extract and isolated theaflavins of Kenyan black tea. Synergism and restoration of activities of lower concentrates of ampicillin, chloramphenicol, tetracycline, norfloxacin and ciprofloxacin by the tea extracts as is shown in this research, a pointer to the value of the blend in combating bacterial resistance.

Black tea and its two polyphenols (theaflavins and thearubigins) have significant antimutagenic effects against *Salmonella* strains (Gupta et al. 2002). Chunxia and Yongquan (2006) also showed that theaflavins have considerable antimutagenic effects against bacterial mutagens such as sodium azide, 4-nitro-o-phenylenediamine, cumine hydro-eroxide, 2-amino-fluorene and danthron. Development of bacterial resistance has been attributed to mutation. Errors in deoxyribonucleic acid (DNA) synthesis during replication and occasional failures in the DNA repair systems result in a spontaneous mutation (Grace 2008). The ability to prevent development of antibiotic resistance is owned by certain antimutagenic agents. These include green tea catechins and other antioxidants. In many cases, these agents can exert these effects at doses which by themselves produce no visible effect on growth. These effects are exerted against resistance to antibiotics such as tetracyclines, fluoroquinolones, macrolides, beta-lactams, and aminoglycosides (Pillai et al. 2001).

Blended use of tea and antibiotics could be useful in erasing the problem of emerging drug resistance especially among enteropathogens. Multi-drug resistance by *S. typhi* was examined. In this research, all 56 isolates of *S. typhi* were sensitive to amoxicillin/clavulanate, gentamicin, cefixime, cefotaxime and ceftazidime. Multidrug resistance (MDR, resistance to three drugs) was found in 22 cases (39%) while resistance to five drugs was found in 12 cases (21%). Only two isolates were resistant to chloramphenicol (3%). All *S. paratyphi* A. isolates were sensitive to ampicillin and chloramphenicol, and resistant to nalidixic acid. Treatment of enteric fever in children based on current trends of antimicrobial susceptibility of *Salmonella enterica serovar typhi* and *paratyphi A* used ampicillin as a chosen drug. MIC distribution data for chloramphenicol revealed elevated MIC, but still in susceptible range. Therefore, recommended an urgent need for further clinical research to evaluate response to chloramphenicol in such cases (Manchanda et al. 2006).

Tetracycline resistance now occurs in an increasing number of pathogenic, opportunistic, and commensal bacteria. The use of these agents in treatment of disease is limited by the presence of tetracycline-resistant pathogens. Tetracycline resistance is often caused by the acquisition of new genes, which code for energy-dependent efflux of tetracyclines or for a protein that protects bacterial ribosomes from the action of tetracyclines (Ian and Marilyn 2001).

Two genetically distinct classes of norfloxacin-resistant *Pseudomonas aeruginosa* PAO4009 mutants were isolated spontaneously. Two norfloxacin resistance genes, nfxA and nfxB, were mapped hex-9001 and leu-9005 and between pro-9031 and ilv-9023, respectively, on the *P. aeruginosa* PAO chromosome. These findings suggested that the norfloxacin resistance mechanism in the nfxB mutant might be an alteration in outer membrane permeability to norfloxacin (Hirai et al. 1987). *P. aeruginosa* has been shown to inactivate anti-methicillin resistant *S. aureus* antibiotics as indirect pathogen (Ramphal 2007).

The blend of hot water extract and isolated theaflavins with antibiotics in this research were found to be useful *in vitro*. However, before utilizing these findings *in vivo*, there are other factors to consider. Black tea has 177-303 mg/l of caffeine. Several drugs can impair the metabolism of caffeine, increasing the potential for adverse effects from caffeine. They include cimetidine (Tagamet), disulfiram (antabuse), estrogens, fluoroquinolones, antibiotics (ciprofloxacin, enoxacin and norfloxacin), fluconazole (diflucan), fluroxamine (luvox), mexilitrine (mextil), riluzol (rilutek), terbinafine (lamisil) and verapamil (calan). High caffeine intakes may increase the risk of toxicity of some drugs, including albuterol (alupent), cloxapine (clozaril), ephedrine, epinephrine, monoamine oxidase inhibitors, phenypropanolamine and theophylline.

Results from this research showed that ciprofloxacin and norfloxacin act synergistically with both tea extracts *in vitro*. This benefit cannot be fully utilized *in vivo* because of the presence of caffeine in hot water extract of black tea. These antibiotics can be blended with decaffeinated black tea and isolated theaflavins. The black tea industry will have to produce another patented tea, in which caffeine has been removed, akin to that of green tea industry.

Flavonoids in tea which includes theaflavins have been reported to bind non-heme iron, inhibiting its intestinal absorption. Non-heme iron is the principal form of iron in plant foods, dairy products, and iron supplements. The consumption of one cup of tea with a meal has been found to decrease the absorption of non-heme iron in that meal by about 70%. To maximize iron absorption from a meal or

iron supplements, tea should not be consumed at the same time (Higdon 2007).

In conclusion**, w**ater soluble components in black tea reduces theaflavins activity but does not remarkably diminish their overall activity. Theaflavins and hot water extracts of black tea can be used to impede growing bacterial resistance despite the interactions. The blended formulations with antibiotics will be suitable for prevention and treatment. Both black tea extracts can restore the activities of antibiotics.

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Fabrication of chitosan-based nanofibrous scaffold using free surface electrospinning for tissue engineering application

PARINITA AGRAWAL, KRISHNA PRAMANIK♥
Department of Biotechnology & Medical Engineering, National Institute of Technology. Rourkela-769008, Orissa, India.
♥email: mondalkp@gmail.com; kpr@nitrkl.ac.in

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Abstract. *Agrawal P, Pramanik K. 2017. Fabrication of chitosan-based nanofibrous scaffold using free surface electrospinning for tissue engineering application. Bioteknologi 14: 60-70.* Tissue engineering offers a promising approach for repair of defective tissues and organs. Developing scaffold from a variety of polymer blends or composites allows the adjustment of the properties aimed at duplicating required tissue. In recent years, considerable attention has been given to develop chitosan-based biomaterials for their applications in the field of tissue engineering due to its minimal foreign body reactions, an intrinsic antibacterial nature, biocompatibility, biodegradability, and the ability to be molded into various geometries and forms such as porous structures that are suitable for cell ingrowth and osteoconduction. The present work involves the preparation of nanofibrous mat from chitosan blended with other biopolymers such as silk fibroin, poly-vinyl alcohol, and polyethylene oxide by free surface electrospinning method. The morphology and functional characterization of the developed scaffolds was performed by SEM and FTIR studies. The average fiber diameter of 269 nm and 122 nm were obtained with chitosan/polyvinyl alcohol and chitosan/silk fibroin, poly ethylene oxide blends respectively. Crystalline nature of the scaffolds was confirmed by XRD studies. The scaffolds are also shown to have desired biodegradable and biocompatible properties. Chitosan-based polymeric scaffolds are thus proved to be potential materials for tissue engineering applications.

Keywords: biocompatibility, biodegradability, chitosan, free surface electrospinning, tissue engineering

INTRODUCTION

Bone and cartilage defects and lesions occur in a variety of clinical situations. Patients are treated in mainly three ways, namely by applying autograft, allograft, or xenograft. Each of these has inbuilt disadvantages, as there is always a chance that the grafted tissue may not work as expected in the patient. Allograft and xenografts have the additional problem of donor scarcity, disease transmission or contamination and immune rejection. Tissue engineering provides a lasting cure for this by offering a biocompatible and replaceable tissue having functional and mechanical integrity (Brahatheeswaran et al. 2011).

Tissue engineering involves scaffold designing including composition, structure, mechanical, biological, and physiochemical features analogous to natural bone and duplicating its extracellular matrix (ECM) (Li et al. 2002; Risbud and Sittinger 2002; Chang et al. 2015). Specific surface area and pore size are important for initial cell adhesion. Improved cell migration provided by scaffolds with pores above 300 microns is significant for scaffold, which was designed for bone or cartilage tissue growth. An added advantage of the larger pores is a reduction in cell aggregations that develop along the edges of the scaffolds. A study by Murphy et al showed that scaffolds with a mean pore size of 325 microns were optimal for bone tissue engineering (Gravel et al. 2006). By facilitating capillary formation, pores greater than ~300 μm lead to direct osteogenesis while pores smaller than ~300 μm can encourage osteochondral ossification. However, larger pores may compromise the mechanical properties of the scaffolds by increasing void volume. Scaffolds for osteochondral tissue regeneration should be non-immunogenic, non-toxic, biocompatible, and biodegradable. The scaffold should possess an interconnected and spread porosity (usually exceeding 90%) with a highly porous surface and microstructure. This would allow in vitro cell adhesion, ingrowth and reorganization and would provide the necessary space for neo-vascularization in vivo. The scaffold should have sufficient mechanical strength during *in vitro* culturing to maintain the spaces required for a cell's growth and matrix formation. Pore size and orientation are shown to influence the mechanical properties of chitosan (CS) scaffolds. Tensile testing of hydrated samples showed that porous membranes have greatly reduced elastic moduli (0.1-0.5 MPa) compared to non-porous membranes (5- 7MPa) (Ji et al. 2006). Moreover, a scaffold must provide sufficient temporary mechanical support and match the mechanical properties to the host tissue as closely as possible; to bear in vivo stresses and loading. It is possible to produce scaffolds with tailored physical, biological, and mechanical properties by combining bioabsorbable polymers and bioactive ceramic phases**.**

A many natural and synthetic polymers have been investigated previously. Chitosan has attracted attention of many researchers because of its characteristic as biodegradable, biocompatible, and non-toxic and thus it has

been believed as a safer material for use in biomedical applications (Hutmacher et al. 2001; Chen et al. 2002; Hutmacher et al. 2004; Cheung et al. 2007). Di Martino et al. (2005) found that CS possesses intrinsic antibacterial activity (Ji et al. 2006). Studies have shown that CS can reduce the infection rate of experimentally induced osteomyelitis by Staphylococcus aureus in rabbits. Its cationic amino group associates with anions on the bacterial cell wall, suppresses the biosynthesis, and disrupts the mass transport across the cell wall. Thus, it accelerates the bacterial death. Due to this antibacterial property, it has been mixed with other polymers in various biomedical related studies. CS has also been reported to be combined with a variety of delivery materials such as alginate, hydroxyapatite, hyaluronic acid, calcium phosphate, PMMA, poly-L-lactic acid (PLLA), and growth agents which are potentially applied in orthopedic tissue engineering.

In recent years, polymer blending has become a method for providing polymeric materials with desirable properties for practical applications. Chitosan blended with PVA has been reported to have good mechanical and chemical properties. Additionally, it has been studied in the biomedical field (Chen et al. 2002). The enhanced property has been attributed to the interactions between chitosan and PVA in the blend through hydrophobic side-chain aggregation and intermolecular and intra-molecular hydrogen bonds.

The other important factor is the fabrication method. While the fabrication of porous scaffold has been the choice of many researchers, the fabrication of scaffold from nanofibres generated by electrospinning is gaining importance in recent years. Electrospinning is a simple and easy way to control the morphology of ultrafine fibers. In this process, high voltage electric is used. The fibers produced by this method have some characteristics, such as very large surface-to-volume ratio and high porosity with a small pore size (Deitzel et al. 2001; Huang et al. 2003), pore distribution is irregular in the matrix. Therefore, there is a need for a systematic research effort to prepare electrospun nanofibres from polymeric blends of chitosan with other biopolymers.

The objectives of this research was (i) to prepare chitosan-based polymer blends of desired properties to develop tissue-engineered scaffold, (ii) to fabricate chitosan-based electrospun nanofibrous mat, (iii) to optimize key parameters of electrospinning process, (iv) To characterize the nanofibrous scaffold, and (v) to perform *invitro* study of cell scaffolding for biocompatibility and biodegradability were the aims of the research.

MATERIALS AND METHODS

Preparation of polymer blend

PVA was dissolved in distilled water (DW) at a concentration of 10 wt%, and chitosan was dissolved in acetic acid-water (AA-water) solution (2 wt%) at a concentration of 2 wt%. These solutions were mixed at different weight ratios of PVA/chitosan, i.e., 90/10, 80/20, 70/30, 65/35, 60/40 and 50/50 (5 mL each).

Preparation of chitosan-silk fibroin blends

Preparation of SF by degumming method

Silk Fibroin (SF) was obtained from *Bombyx mori* silkworm cocoons by the Degumming method, which includes cutting the cocoons into small pieces, cleaning and removing completely the traces of the silkworm and any other debris. The cocoons were then washed with distilled water and then boiled in 0.01 M sodium carbonate for 60 min; then they were washed under running distilled water thrice to remove sericin. After an overnight oven drying at 45°C, the resultant fibers were dissolved in 9.3 M Lithium bromide (LiBr) and heated at 50°C. LiBr residue was removed by dialysis process, using dialysis cassette (Thermo Scientific, slide-A-Lyzer 10K) against distilled water for three days, with the water being changed every 3h. The dialyzed solution was freeze-dried in a lyophilizer to obtain silk in dried powder form (now onwards referred as regenerated SF). The regenerated SF powder was kept in airtight container until it was needed.

Silk fibroin solution preparation and blending with CS

Regenerated silk fibroin powder was dissolved in the aqueous solution to form 1 wt% polymer solution. The solution was mixed and allowed to stir for 24 hours. CS/SF blend solutions were prepared by mixing CS and SF solutions in different ratios by volume (75:25, 50:50, 25:75 and 10:90) making final volume to 5 mL. These solutions were kept on magnetic stirrer overnight, after which they were electrospun.

Preparation of chitosan-SF-PEO blends

Polyethylene oxide (PEO) powder was added to the CS/SF blend solutions to modify them and enhance the fiber formation efficiency during electrospinning. Thus CS, regenerated SF and PEO powder was mixed in weight ratios 1:1:1, 2:1:1 and 2:2:1 (CS:SF:PEO) and stirred overnight , before being subjected to electrospinning.

Study of Rheological behavior of polymer blends

Prior to electrospinning, the viscosity of solutions was tested by Bohlin Visco 88 viscometer, manufactured by Malvern Instruments, U.K. To calculate viscosity, Moore Model was applied.

Preparation of nanofiber by electrospinning

Nanofibers were made by subjecting polymers to high voltage in electrospinning machine (Elmarco, Nanospider "NS Lab 200"). The samples were tested for fiber formation by keeping a drop of the sample on the sample space under various process conditions like changing electrode to collector distance (working distance), the voltage applied and electrode rotation speed. Those blend ratios which were able to form fibers were then electrospun in higher volumes for obtaining nanofiber sheets. The fibers were sorted and collected on the fabric after drying which was then stored for characterization.

Study of key electrospinning parameters

Ratio of polymers in the blend

Polyethylene oxide is added to the blend solution to make the CS:SF formulation electrospinnable. PEO is a synthetic polymer, thus its degradation and removal from the body is an issue when used in larger amounts. Lowering the amount of PEO will serve the purpose of decreasing immune reaction when the cell-scaffold construct is incorporated into the body.

Weight ratios of CS:SF:PEO were prepared to keep minimum possible ratio of PEO. CS:SF:PEO (1:1:0.4, 1:1:0.3, 1:1:0.2 and 1:1:0.1) solutions were prepared and kept for stirring overnight; then these were electrospun to check nanofiber formation.

Effect of process parameters

For testing optimum process parameters, the electrospinning was performed under various processing conditions, namely the applied voltage (working distance) and the speed of electrode rotation (rpm).

Characterization of nanofibrous scaffold

Morphology analysis

The SEM (Scanning Electron Microscopy) was used to evaluate the morphology and microstructure of the synthesized samples. The electrospun fiber samples were coated with a thin layer of platinum (Pt) and their morphologies were observed under a Scanning Electron Microscope (JEOL-JSM 6480 LV SEM), that was operated at the acceleration voltage of 15 kV. Images were taken at 5000X, 10000X and 20000X magnifications.

XRD analysis

The electrospun fibers were subjected to X-rays to obtain a X-ray diffraction (XRD) pattern to reveal detail information about the chemical composition and crystallographic structure of manufactured nanofibres. The instrument used for scanning was XRD- PANalytical and range were 10^{o}-50^{o} keeping the 2-theta step size.

FTIR analysis

Fourier Transform Infra-Red (FTIR) was used to characterize molecular structure of nanofibers. FT-IR analysis was based on the identification of absorption bands due to the vibrations of functional groups presented in macromolecules (Tangsadthakun et al. 2006).

To make a pellet, Nanofibrous polymer scaffold was grounded into a fine powder. A thin fiber sheet was then pressed in between the two KBr powder layers in the KBr press Technosearch instrument. This preparation was then pressed from 0-10 tons and then released. By this process, a pellet was formed. This pellet was then placed in IR-Prestige-21 to record the FTIR readings, and a plot of wavenumber (cm-1) versus percent transmittance (%T) is prepared.

Swelling ratio and water uptake capacity

The conventional gravimetric method was used to measure the equilibrium swelling ratio (Es). First, the dry weight (Wd) of the scaffold was measured and then, it was immersed in distilled water and incubated for 24h at 37^{o}C. The wet weight (Ws) of the scaffold was determined by weighing it after the excessive water from the immersion was blotted out with absorbent paper. The equilibrium swelling ratio of the scaffolds was defined as the ratio of weight increase (Ws-Wd) about the initial weight (Wd) of dry samples. Each value was averaged from three parallel measurements. Es was calculated using the following equation:

$$Es = (Ws-Wd)/Wd$$

And water uptake percentage (Wu) was measured using the equation:

$$Wu = (Ws-Wd)/ Ws \times 100$$

Mechanical strength testing

Sample preparation for tensile testing. Fiber sheets were cut into specific geometry, namely 20mm X 10mm, and a cardboard sheet was pasted at each end to provide support and grip of the clamp. The thickness of the sheets was measured using Digital Vernier Calpier (Absolute Digimatic, Mitutoyo).

Tensile test. Tensile strength of electrospun nanofiber sheets was measured using Universal Mechanical Tester. The fiber sample was stretched with a computer controlled Instron Electropulse E1000 to test its tensile strength. After a particular load and elongation, the sample breaks, and the program generates the result in the form of graph of Load v/s Extension. Depending on the fed information regarding dimensions of the sample and the generated raw data by the program, several parameters are also shown in the result: like load at break, Modulus, and tensile strength of the sample. To ensure a reliable result, the process was performed twice for each sample.

Biodegradation study

The scaffolds with dry weights noted were sterilized by immersing in 70% ethanol and then in stimulated body fluid (SBF) with pH 7.4 at 37°C. The SBF solution was replaced daily to ensure continuous degradation. Samples were removed from the medium, rinsed with distilled water and weighed in every 15 minutes for the first hour and then every 2h for 24h and then twice regularly for one month. The experiment was done in triplicates for each scaffold. The extent of degradation was expressed as a percentage of weight remained of the dried sample after degradation. The percentage of weight loss was calculated using the following equation:

$$\text{Weight loss} = (Wi - Wf)/Wi \times 100$$

Where, Wi and Wf represent the initial and final weight of scaffolds, respectively.

***In-vitro* biocompatibility study**

To study the biocompatibility of electrospun nanofibres, the cells were seeded on the scaffold. Following steps were performed for this: (i) Scaffold sterilization- Electrospun

nanofibres were sterilized by immersing them in 70% ethanol for 1 h. (ii) Scaffold neutralization- Scaffolds were neutralized by washing them with PBS 3-4 times at regular intervals. The pH of the solution was also checked every time PBS was changed. When the pH was close to 7, the scaffold was neutralized. (iii) Cell preparation- Mesenchymal stem cells were trypsinized and suspended in DMEM and 10% FBS having broad-spectrum antibiotic was centrifuged to obtain individual cells in a suspension. (iv) Cell seeding- Cells were seeded on the sterilized nanofibers and kept for incubation at 37ºC for 72 hr.

RESULTS AND DISCUSSION

Preparation of polymer blend

Polymer solutions were mixed in different w/w and v/v ratios and stirred overnight on magnetic stirrer, to form clear blends, which were then characterized and processed to form nanofibres.

Study of rheological behavior of polymer blends

The viscosity of pure Chitosan was 0.317 Pa sec which increased several folds after being blended with PVA solution at various ratios, as shown in Figure 1. This is in line with the data published by Paipitak et al. (2011), who reported that a linear increase in viscosity of CS solution blending with increasing amounts of PVA. Blending CS with SF and PEO also showed increment in viscosity, as shown in Figure 1.

Experiments performed by Alhosseini et al. (2012) have established that the high viscosity increases the interaction of two polymers, mainly through hydrogen bonding, and decreases the effects of surface tension. This will result in formation of fibers with uniform morphology after electrospinning.

Study of key electrospinning parameters

Ratio of polymers in the blend

CS:SF:PEO blend solutions were prepared by keeping the minimum possible ratio of PEO and electrospun to check nanofiber formation. No fiber was formed for the blends containing less than 0.5 ratios of PEO. The result is shown in Table 1.

Effects of process parameters of electrospinning

The parameters studied for optimization are listed in Table 2.

Characterization of nanofibrous scaffold

Morphology analysis-Scanning Electron Microscopy (SEM)

CS/PVA nanofibres- Figure 2.A-J shows SEM micrographs of the electrospun CS/PVA nanofibers. An average fiber diameter of CS/PVA blend, weight ratio 90/10 was found to be 300 nm with a range of 240-349 nm. For blend ratios 70/30, 65/35 and 60/40, the average fiber diameter obtained was 282 nm, 264 nm, and 260 nm respectively. The trend of decrease in fiber diameter with decreasing PVA concentration in the blends was observed, and with the blend ratio 50/50 (CS/PVA) resulted in fibers with beads morphology (Figure 2.I-J).

CS/SF/PEO Nanofibres- Morphology analysis of CS/SF/PEO mixed scaffolds showed unaligned nanofibers formation as showed in Figure 3.A-D. Micrograph of ratio 1:1:1 scaffold at 20,000X magnification suggests that the diameter of the fibers was less various and was within the range of 122nm to 130nm. For combination ratio 2:2:1, the fiber diameter was found to be in the range of 120nm to 126nm.

In electrospinning, the fiber diameter is dependent on the viscosity and charge of the solution. It was discovered that fiber diameter increased as the viscosity rate increased. CS affects not only the viscosity, but also the rate density at the surface of the ejected jet through its cationic polyelectrolytic property. It increases the rate density on the surface of the jet, which in turn increases the elongation pressure and reduces the diameter of the fiber (Desai et al. 2009).

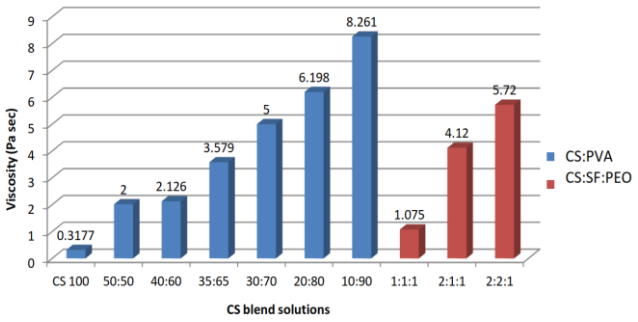

Figure 1. Viscosity measurement of CS blend solutions.

Table 1. Effect of composition of polymer blend on electrospinning.

CS:SF:PEO blend	Nanofiber formation
1:1:0.1	No
1:1:0.2	No
1:1:0.3	No
1:1:0.4	No
1:1:0.5	Yes
1:1:1	Yes

Table 2. Effect of Applied voltage, Working Distance, and electrode rotation on electrospinning.

Sample	Voltage applied (kV)	Working Distance (cm)	Electrode rotation speed (rpm)
CS:PVA	70	11.5	6.8
CS:SF	15 to 50	11.5	7.0
CS:SF:PEO	60	12.0	7.0

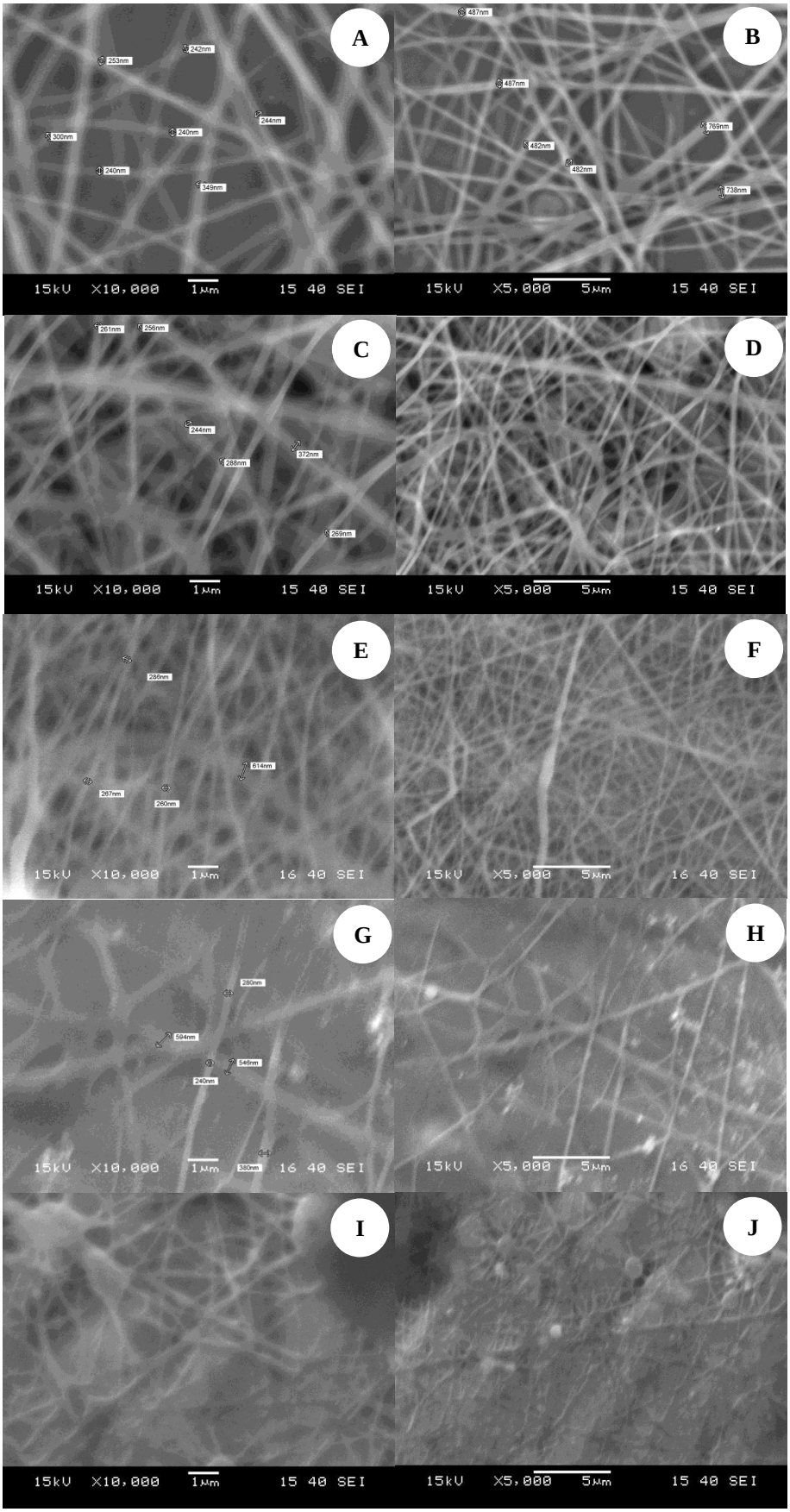

Figure 2. SEM micrograph of electrospun CS: PVA fibers of ratio 10:90 (A-B), 30:70 (C-D), 35:65 (E-F), 40:60 (G-H) and 50:50 (I-J).

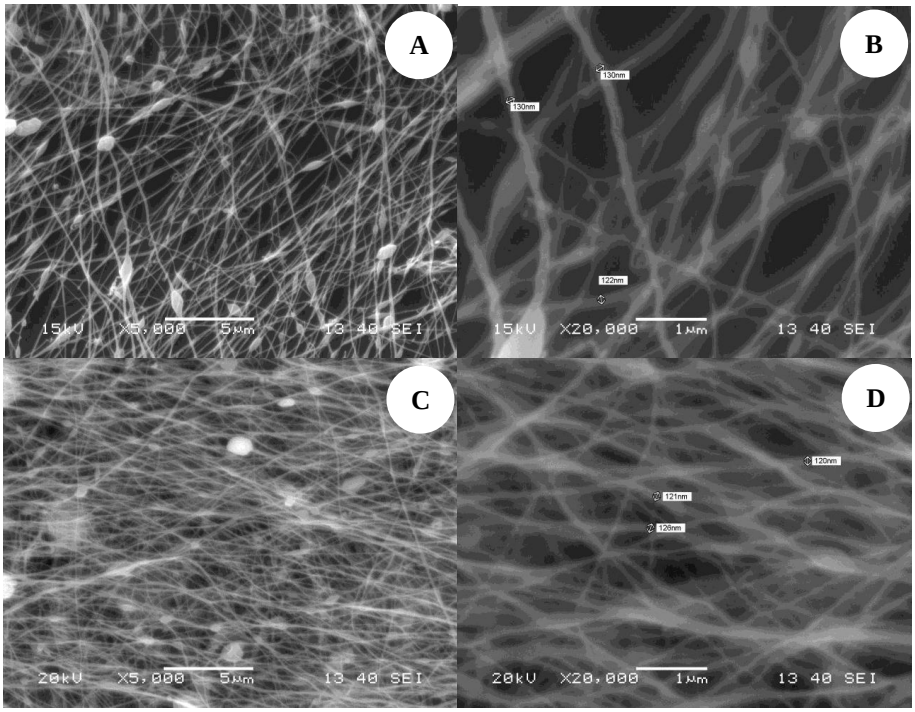

Figure 3. SEM micrograph of electrospun CS:SF:PEO fibers of ratio 1:1:1 (A-B) and 2:2:1 (C-D).

Table 3. Standard X-Ray Diffraction peak values for polymers used

Component	2 Theta value of	
	Major peak	Minor peak
Chitosan (CS)	19o	9.4o
Polyvinyl alcohol (PVA)	30o	48o
Silk Fibroin (SF)	20.4o	24.5o
Polyethylene oxide (PEO)	30o	40o

Phase analysis

X-ray diffractogram was obtained and shown in Figure 4 and Table 3. This was used for analyzing for accessing the crystallinity of the sample. Phase change during blend formation and electrospinning process were studied by XRD.

CS/PVA nanofibres- A dome-shaped curve at 20º in 35:65 (CS:PVA) sample depicted the presence of chitosan in it, while peaks at 30º and 48º confirmed the presence of PVA in all the prepared composites (Figure 4.A).

CS/SF/PEO Nanofibres- Rise at 20º confirmed the presence of chitosan in the sample and domes near 25º showed the presence of silk fibroin in the blends. Both the blends showed all the major and minor peaks of PEO in the X-ray diffractogram (Figure 4.B).

Hence, it was noted that the stage of the blends does not alter after processing, and the diffractogram also confirmed the availability of all the components in the blends along with their crystalline nature when processed into a scaffold by electrospinning. Crystallinity alludes to the degree of structural order in a solid. In a gem, the atoms or molecules are orchestrated in a regular, but by a regular, occasional way. Polymer shape crystalline regions have generally long lengths of molecules that usually prevent complete crystallization. Numerous polymers show semicrystalline behavior.

FTIR analysis

The inter-molecular interaction can be distinguished by FTIR, when two polymers are blended for nanofibres fabrication (Tangsadthakun et al. 2006). In the case of a CS-PVA blends and CS, SF and PEO composites used for electrospinning of nanofibers, the FT-IR analysis was based on the recognition of absorption bands concerned with the vibrations of functional groups present in macromolecules. FT-IR spectra obtained from pure chitosan, Chitosan/PVA, and pure PVA films are shown in Figure 5.A and for CS/SF/PEO films are shown in Figure 5.B.

For the spectrum of pure chitosan as seen in Figure 6, the characteristic absorption bands of chitosan were observed at six locations. The vibrations of hydroxyl and free amine groups appeared at 3439 and 3300 cm- 1, respectively. The absorption bands at 1655, 1560 and 1381 cm-1 indicated C=O stretching, -NH2 bending and C-O stretching of primary alcohol groups, respectively. The last one at 1152 cm-1 represented -C-O-C- glycosidic linkage between chitosan monomers (Miya and Iwamoto 1984).

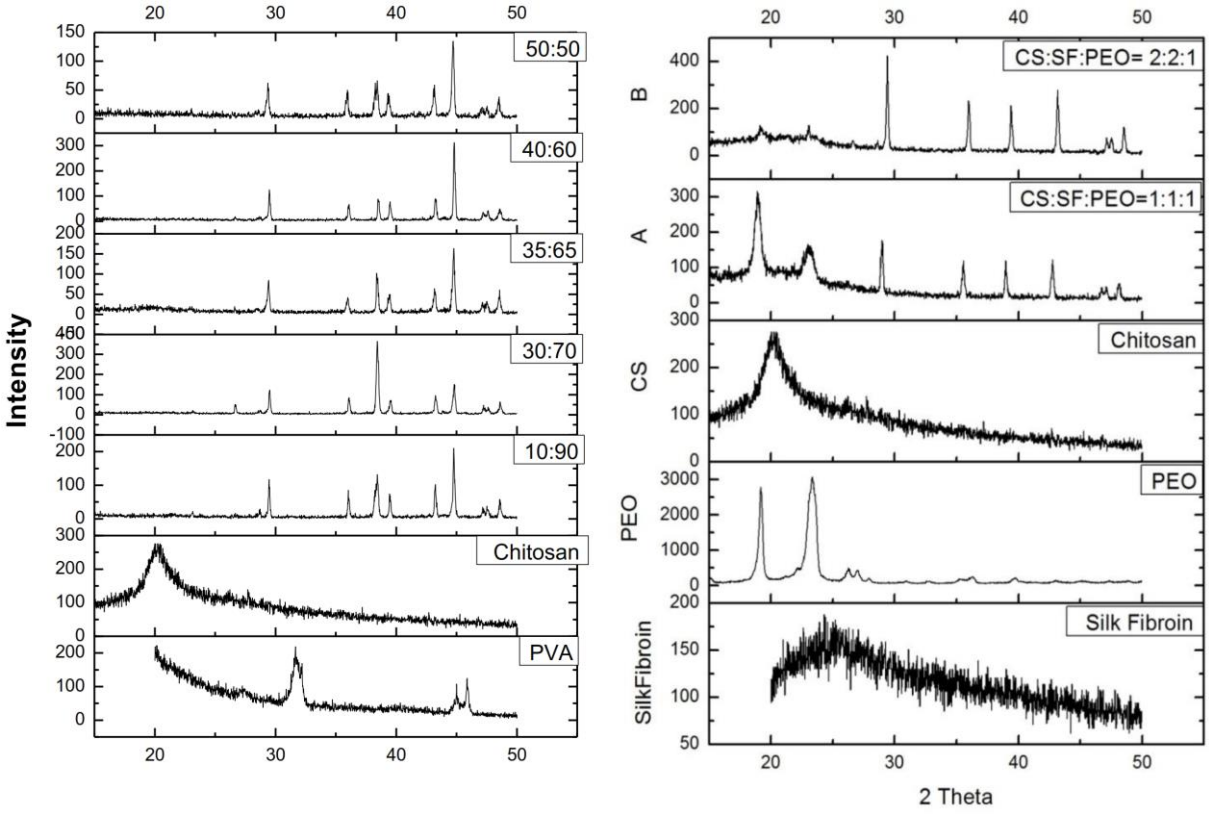

Figure 4. XRD analysis of electrospun CS:PVA blends (A) and CS:SF:PEO blends (B).

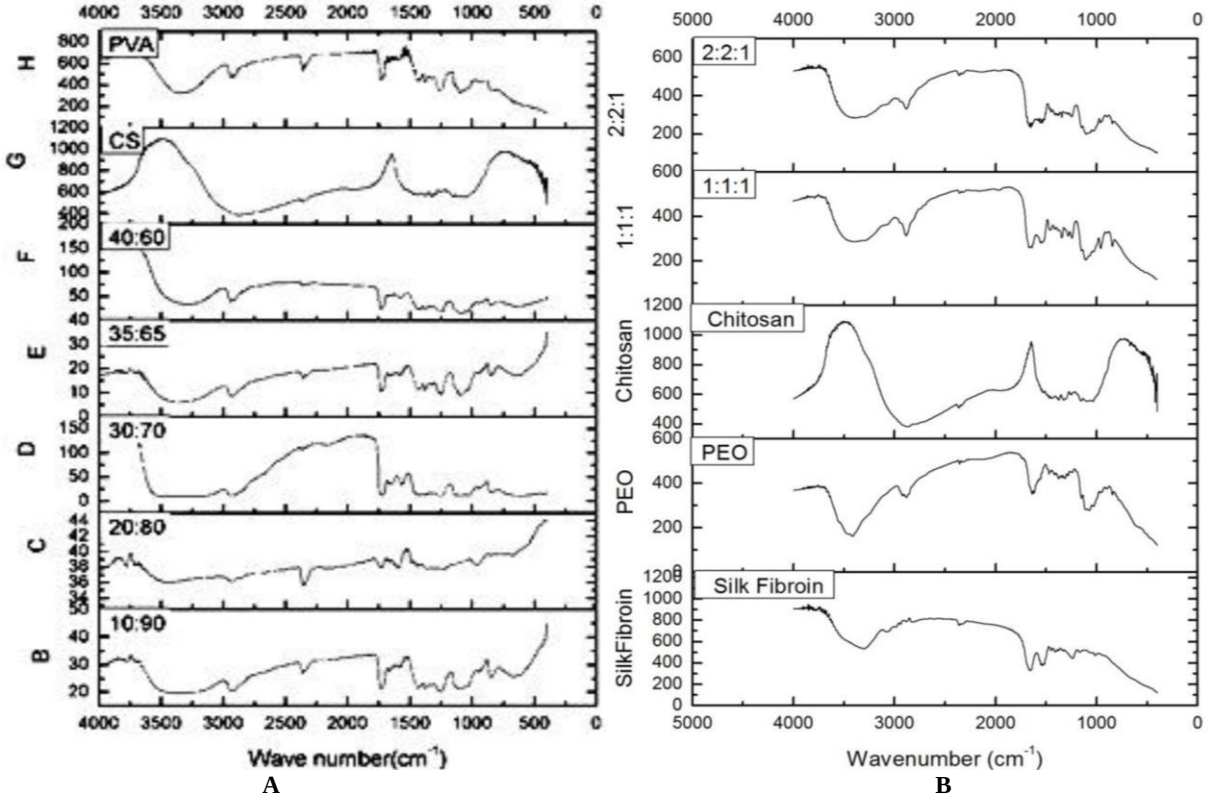

Figure 5. FTIR spectra of CS/PVA (A) and CS/SF/PEO (B) blends.

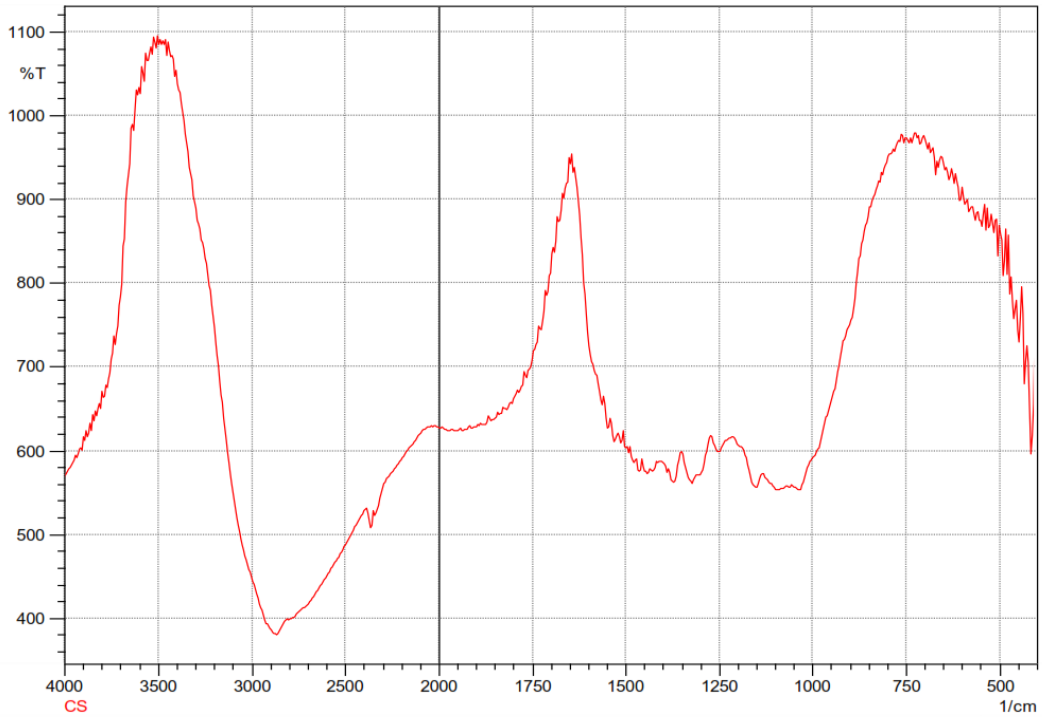

Figure 6. FTIR spectrum of Chitosan.

CS/PVA blends- In FTIR spectra of PVA, all major peaks related to hydroxyl and acetate groups were examined. Large bands between 3550 and 3200 cm-1 are linked to the stretching O-H from the intermolecular and intramolecular hydrogen bonds. The vibrational band between 2840 and 3000 cm− 1 refers to the stretching C-H from alkyl groups and the peaks between 1750-1735 cm− 1 are due to stretching C O and C-O from acetate group. The shift in the lower order of spectrum for the Chitosan/PVA blends is mainly due to primary alcohol and secondary alcohol interactions, which took place due to hydrogen bonding as it is earlier reported in studies of chitosan and PVA blends (Miya and Iwamoto 1984; Young et al. 1996).

CS/SF/PEO blend- FTIR spectra of pure SF illustrated four characteristic absorption bands for silk fibroin. At 700 cm− 1, it was due to amide-V group vibration while at 1260 cm− 1, it was due to amide III vibration and was presented in random coil of the structure. Amide II group present on beta-sheet conformation of SF showed absorption at 1525 cm− 1 position. While band at 1625 cm− 1 can be attributed to C O bond vibration or if the molecule is in β-sheet conformation, then this band depicts amide-I bond vibration. For the spectrum of pure PEO, various band locations that signify functional group are at 841 cm− 1 and 961 cm− 1 C-H2, O-C-O bond stretching is depicted at 1101 cm− 1 and band at 2891 cm− 1 was linked to C-H bond stretching.

Swelling ratio and water uptake capacity

CS/PVA blends- As shown in Figure 7, the swelling behavior of CS/PVA scaffolds with different ratios and time could be clearly distinguished. The blends containing CS less than 30% (w/w) showed good swelling. The other group of which the swelling ratios were as low as that of pure chitosan was the blends having chitosan composition more than 30%. This phenomenon can be attributed to the loss of gel-like structure after swelling. Water intake capacity of all the chitosan/PVA blends was approximately same, and the average value was 98.5576%.

CS/SF/PEO blends- Weight of CS/SF/PEO blends were found to be lesser than 30% of swelling. Water uptake percentage was also lesser than CS/PVA blends.

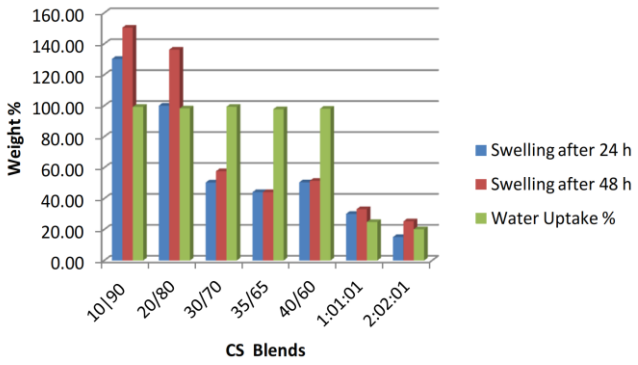

Figure 7. Swelling Ratio and Water Uptake Capacity of Chitosan composite scaffolds.

Mechanical testing

Due to the tiny dimension, the mechanical characterization of an individual nanofiber is a challenge for the existing test techniques. Figure 8 shows typical stress-strain curves of CS- composite nanofibers obtained by electrospinning for tissue engineering applications. Table 4 summarizes the tensile strengths obtained from different scaffolds composition at various loads. It was concluded that the tensile strength of the nanofibers largely depends on their geometry and composition.

CS/PVA blends- A trend of increase in tensile strength was in accordance with the increase in CS composition in the blends. The break at stretching which can be seen in each case, followed the trend made by non-uniform sheets. In the case of 40:60 nanofiber sheet, lesser thickness value (0.02mm) limited its tensile testing by this method.

CS/SF/PEO blends- Nanofiber sheets comprising the components in 1:1:1 ratio showed less tensile strength, and 2:2:1 composition of nanofiber sheet was so thin that its tensile testing could not be done by this method. It can be concluded that the presence of SF in the composite makes the fibers comparatively brittle, and thus decreases the tensile strength.

Non-uniform break on stretching can be explained by this; samples comprise of unaligned nanofibers, as revealed by SEM micrographs, thus the force gets distributed into many directions during stretching, while on the aligned fibers, the distribution of force is in a particular direction resulting into straight cut, as shown in Figure 9.

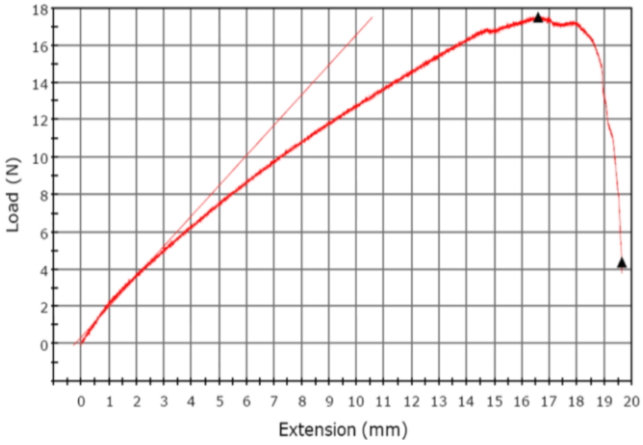

Figure 8. Stress-strain curve of CS composite Nanofibers.

Table 4. Tensile strength of composite scaffold at varying load.

Sample label	Sample thickness (mm)	Maximum load (N)	Tensile strength (MPa)	Load at break (N)	Tensile strain at break (%)	Modulus (kPa)
CS:PVA						
10:90	0.10	5.79	3.22	1.44	38.91	23408.32
20:80	0.30	13.98	4.66	4.77	109.52	7572.45
30:70	0.25	5.93	5.93	5.84	25.67	37687.91
35:65	0.31	18.44	6.15	10.49	83.44	12385.66
40:60	0.02	--	--	--	--	--
CS:SF:PEO						
1:1:1	0.055	2.89	5.79	2.77	1.36	425990.53
2:2:1	0.02	--	--	--	--	--

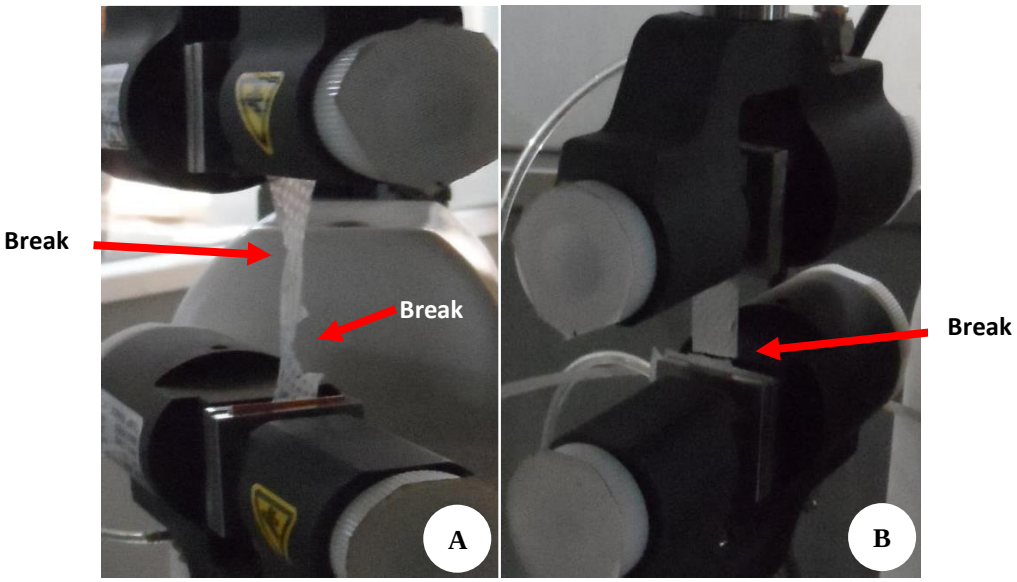

Figure 9. Stretching and break of un-aligned (A) and aligned (B) fiber sheets

The developed strategies and standards for figuring out the mechanical behavior of conventional fibers are insufficient in the case of manipulating or testing of nanofibers. It has been found that tensile strength of nanofibrous mat became less than that of cartilage, which should be near 40 MPa. This experiment was executed on non-aligned fibers, for fiber orientation plays an important role in determining tensile strength of any material. Fibers show good tensile strength when it is pulled along the path of fibers. Consequently, an attempt to form properly aligned nanofibers might comply the desirability in attaining the favored mechanical strength (Figure 10).

Biodegradation

The biodegradation results are shown in Figure 11 and 15.

CS/PVA blends- PVA scaffolds incubated in SBF had the highest weight reduction and were absolutely degraded after 30 mins. However, the addition of chitosan decreased the degradation of scaffolds in SBF solution. Concerning the stableness of scaffolds which was higher than that of pure PVA scaffolds, the obtained results proved to be the crucial feature, for it is known that the degradation rate of PVA scaffolds can be quick. Therefore, the addition of chitosan could extend the biodegradability of scaffolds. Most scaffolds showed complete degradation within 30 days of observation. While the blend ratio of 30:70 and 35:65 (CS: PVA) was disintegrated into smooth smaller pieces , of which weighing became difficult beyond 30 days.

CS/SF/PEO blends- Scaffolds were incubated in SBF, and it gave an understanding that degradation commenced immediately on the first hour and then there was no considerable change for the remaining 24hr. Their degradation was resumed after that and followed by weight loss, and the scaffolds were completely degraded on sixth day of degradation study.

I*n-vitro* biocompatibility study

Cells did not connect to the scaffold surface. Cell development was not observed in the arranged scaffolds. The media might lack adequate growth factors for cell attachment, growth, and proliferation. The scaffold might not have been neutralized since the utilization of acetic acid as solvent has made the formulation more acidic and thus it made the formulation unsupportive for cellular growth.

In conclusion, chitosan was mixed with PVA and SF polymers. The polymer mixes were effectively electrospun to manufacture CS/PVA and CS/SF/PEO nanofibrous platforms. The CS/SF/PEO scaffolds have been found to show superior physicochemical properties, compared with CS and CS/PVA platforms. Efforts to move forward mechanical properties of CS-based composites are basic for its application in bone tissue building. Cell study has affirmed that these mixes have beneficial biological properties, like biocompatibility and biodegradability. The spreading of cells on the scaffold surface was a bit nonuniform, as studied by preparatory cell culture research for which detail study is required to indicate their use for cell types. Chitosan can be mixed with numerous polymers as executed in present research; its mixing with ceramics can be studied to manufacture a more potent material which could be used in tissue engineering for precise tissues or cell types. Detail in vitro cell growth is required to ascertain the scaffold for precise tissue regeneration (Table 5).

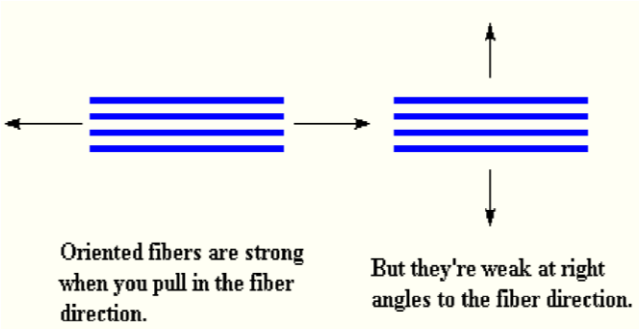

Figure 10. Relation between fiber orientation and tensile strength.

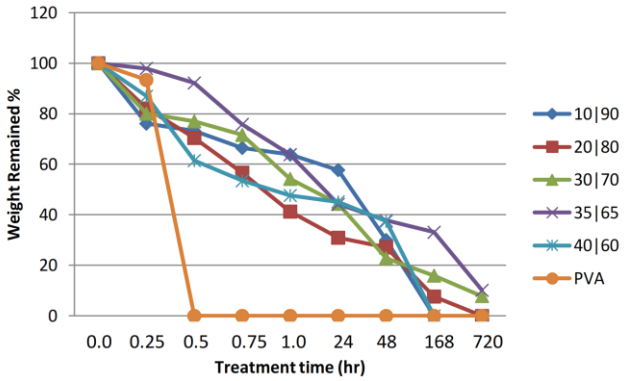

Figure 11. In vitro biodegradation of CS/PVA scaffolds with different blending compositions.

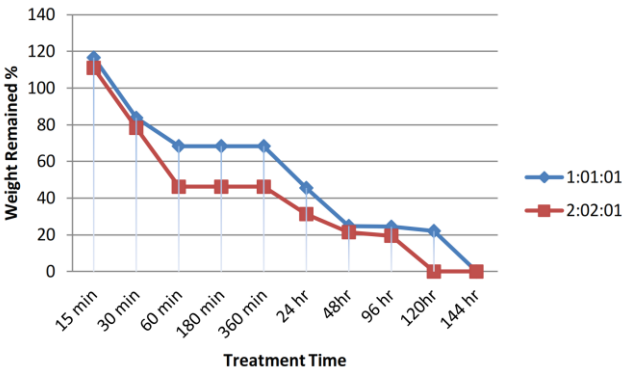

Figure 12. In vitro biodegradation of CS/SF/PEO scaffolds with different blending compositions.

Table 5. Combined results for various characterization techniques

Characterization Technique	Property of comparison	CS/PVA blends	CS/SF/PEO blends	Remarks
Rheology behavior	Viscosity Measurement	2 Pa sec - 8.261 Pa sec	1.075 Pa sec - 5.72 Pa sec	Increasing with increasing ratio of PVA
Morphology Analysis (SEM)	Fiber Diameter (Average)	300 nm	120 nm	Addition of SF results into finer nanofibers
X-ray Diffraction (XRD)	Diffraction pattern and phase change	No phase change	No phase change	Composites contain the components blended prior to electrospinning
FTIR	Functional group detection	Present	Present	Composites contain functional groups of their pure form components
Swelling Ratio	Swelling in weight %	Good swelling observed (~ 88.03%)	Lesser swelling (~29.202%)	Best result for CS/PVA 10:90 and 30:70
Water Uptake capacity	Water uptake Percent (Hydrophilicity)	(98.5576%)	Lesser uptake (22.53%)	CS/SF/PEO blends are less hydrophilic than CS/PVA blends
Mechanical Testing	Tensile strength	3.22- 6.15 MPa Average 4.99 MPa	5.79 MPa	Nanofibers formed by electrospinning possess considerable load-bearing capacity
Biodegradation	Weight loss in SBF wrt time	Complete Degradation after 30 days	Complete degradation in 6 days	Weight loss rate of CS/PVA scaffold is lower than that of CS/SF/PEO

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