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**Tables** should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

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Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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*Full Length Research Paper*

# Guttation fluid as a physiological marker for selection of nitrogen efficient rice (*Oryza sativa* L.) genotypes

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**A field experiment was conducted during the rainy season of 2008 and 2009 to use guttation fluid as a physiological marker for the screening of more nitrogen efficient rice genotypes on the basis of relationship between Guttation Fluid (GF) oozed by leaf tip and nitrogen use efficiency (NUE), grain yield (GY) and biological yield (BY) amongst five rice (KRH-2- hybrid, Kasturi- aromatic, Krishna Hamsa, Tulsi and Vasumati- high yielding) genotypes grown at four nitrogen levels (0, 50, 100 and 200 kg ha$^{-1}$) in alluvial soil of Pantnagar (Uttarakhand), India. The nitrogen fertilizer (urea) was sprouted in the field. For this experiment, the field was made to keep with 5 cm standing water throughout active tillering and reproductive stage. Guttation fluid (GF) was collected during flowering stage whilst other traits after harvesting of the crops. The utmost and lowest GF was achieved by genotype KRH-2 and Kasturi respectably. All the rice genotypes showed the positive correlation between GF and NUE, GY, BY at different nitrogen levels vice-versa. The KRH-2 illustrated better response to secretion of guttation fluid and other traits. The experiment concluded that the amount of GF is directly associated with application of fertilizer as well as NUE, GY and BY. Further studies are good opportunities for rice researcher to improve rice yield through this way and mapping the genes controlling this trait and creating rice plant with increase guttation fluid at different nitrogen levels for selection of high nitrogen efficient rice genotypes.**

**Key words:** Biological yield, guttation fluid, nitrogen use efficiency, grain yield, rice genotypes.

## INTRODUCTION

Rice is one of the most important crops of the world and forms the staple diets of about 2.7 billion people and it needs to be produced 50% more than what is produced now by 2050 to cope with the growing demand (Ashikari et al., 2005). In Asia, 90% of the world's rice is produced and consumed. It is very well known fact that stability and growth in current and future scenario of every nation is only achievable by using their resources efficiently and effectively to meet the demand of rapidly growing populations (Ahmad et al., 2009). The yield of rice is an integrated result of various processes, including canopy photosynthesis, conversion of assimilates to biomass, partitioning of assimilates to grains (harvest index) and interception of light by leaf surface area. Grain weight is an important yield component in cereal crops. It is determined by the source capacity (photosynthetic leaves) to supply assimilate during the ripening period, and by sink capacity (developing grain) to accumulate the imported assimilate. Cultivars with larger grain size tend to have higher grain filling rate, resulting in higher assimilate accumulation and heavier grain weight (Kropff et al., 1994; Zhang et al., 2004; Yoshida et al., 2006;

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**Table 1.** Some basic properties of soil from experimental site.

| Soil properties | Values |
|---|---|
| Soil colour | Dark grayish brown to dark grey |
| Texture | Loam to silty clay loam |
| Soil water (dry mass percentage of water) | 13.99 to 19.78 (June to September) |
| Temperature of soil | 26 to 32°C (June to September) |
| Bulk densities | 1.10 to 1.46 mg $m^{-3}$ (June to September) |
| Hydraulic conductivity | 317 to 407 mm $h^{-1}$ |
| Infiltration rate | 269 to 624 mm $h^{-1}$ |
| pH | 6.74 to 8.05 |
| EC | 0.33 to 0.60 $dSm^{-1}$ |
| $CaCO_3$ | 0.42 to 0.87% |
| CEC | 8.1 to 18.22 meq 100 $g^{-1}$ soil |
| Organic carbon | 0.39 to 1.61% |
| Zn | 0.17 to 2.11 mg $kg^{-1}$ |
| Cu | 0.64 to 2.49 mg $kg^{-1}$ |
| Mn | 1.31 to 45.69 mg $kg^{-1}$ |
| Fe | 3.09 to 21.41 mg $kg^{-1}$ |
| Total N | 0.14% |
| Available P | 44.28 ppm |
| Available K | 280.59 kg $ha^{-1}$ |

Yang et al., 2005). In addition to this, nitrogen is the key factor which limits the yield of rice production around the world. The predominant form of nitrogenous fertilizer applied to the soil is urea. In agriculture, 60% nitrogen is used for cultivation of cereal crops alone, out of 60% approximate 30% of applied N is utilized by crops and the remaining 70% of it is lost through various process causing serious environmental problems (Jiang et al., 2005). Thus, efficient utilization of nitrogen fertilizers is essential for ensuring better yield as well as to minimize the adverse impacts of nitrogen species in the environment.

Nitrogen use efficiency varies amongst rice genotypes due to genetic variation in uptake of nitrogen. High nitrogen application increased the nitrogen uptake but if the application exceeds optimum value, the yield decreased (Wilson et al., 2006). The NUE values ranged from 35.6 to 51.6% for different genotypes (Samonte et al., 2006). Various strategies are being used to enhance yield of the rice crop worldwide such as the transfer of $C_4$ trait to $C_3$ plants for improving the photosynthetic performance of $C_3$ plants (Miyao, 2003). Beside this, another alternative approach for improving rice yield is the selection of varieties those secreting more amount of guttation fluid which is non-invasive, simple, accurate and quick to perform. Guttation fluid is also a potential yield enhancing trait in rice. This guttational fluid contained various organic and inorganic substances of metabolic significance. Ozaki and Tai (1962) found three peptides in the guttation liquid of rice seedling and considered that peptides might be predominant form of nitrogen transport in the xylem of rice seedling. The rates of guttation fluid positively correlated with their panicle weight or grain yield productivity (Singh et al., 2008, 2009).

The rice leaves secrete guttation fluid through openings situated along the leaf margin and tips called hydathodes. The rates of guttation exhibited by various cultivars were positively correlated with their panicle weights, that is, yield sink potential, which is a direct measure of grain yield productivity. The amount of guttation fluid is directly associated with yield of rice crop. The hybrid and heigh yielding rice genotypes show better response to secretion of guttation fluid from leaf tips (Singh et al., 2008, 2009). Now its time to use guttation fluid as a physiological marker for screening of more nitrogen use efficient as well maximizing grain yield to avoid maximum application of nitrogen fertilizer for cultivation of rice crop. Therefore, this study will be benefitted in future for selection of high yield and more nitrogen efficient genotypes using guttation fluid as a physiological marker.

## MATERIALS AND METHODS

The experiment was carried out at Dr. N. E. Borlaug's Crop Research Center (29° N latitude, 79° 29′ E longitude and at an altitude of 243.8 msl.), Department of Plant Physiology, G. B. Pant University of Agriculture and Technology, Pantnagar (Uttarakhand), India, during *kharif* season 2008 and 2009 with five rice genotypes, namely KRH-2 (hybrid), Kasturi, Krishna Hamsa, Tulsi (high yielding) and Vasumati (aromatic) with four nitrogen levels (0, 50, 100 and 200 kg N $ha^{-1}$). Some basic properties of soil from experimental site are shown in Table 1. The field experiment was laid out in a split plot design with 3 replications. The sub-plot size

**Table 2.** Effect of N levels on guttation fluid (µl/tip) of five rice genotypes.

| Genotype | Guttation fluid (µl/tip) | | | | |
|---|---|---|---|---|---|
| | Flowering stage | | | | |
| | $N_0$ | $N_{50}$ | $N_{100}$ | $N_{200}$ | Mean |
| KRH-2 | 97 | 110 | 135 | 163 | 126 |
| Vasumati | 95 | 106 | 119 | 146 | 116 |
| Kasturi | 90 | 104 | 122 | 142 | 114 |
| Tulsi | 93 | 108 | 125 | 150 | 119 |
| Krishna Hamsa | 101 | 114 | 130 | 154 | 124 |
| Mean | 95 | 108 | 126 | 151 | |

| | Treatment (T) | Variety (V) | T × V |
|---|---|---|---|
| S.E.M. ± | 0.29 | 0.33 | 0.67 |
| CD ($p$ = 5%) | 1.01 | 0.97 | 1.95 |

was 4 × 4 m. The 25 days old seedlings were transplanted with 20 cm row space and at 10 cm plant to plant distance. Nitrogen fertilizer in the form of urea was sprouted in three splits as 50% at 15 days after transplanting, 25% at panicle initiation and remaining 25% at the time of flowering stage. The field was made to keep with 5 cm standing water during active tellering and reproductive stage. The bund (approximate 1 m thick and 0.7 m height) was prepared around entire plot as well as sub plots to avoid the movement of applied fertilizer and water to adjacent plots.

The guttation fluid from the flag leaf of individual genotypes at flowering stage was achieved using method described by Singh et al. (2008) and amount expressed in µl/tip. Little modifications were made in this method. The Hamilton syringe and eppendorf tubes were used to collect guttation fluid from the leaf tip instead of blotting paper used in the original method. The guttation fluid from the flag leaf of individual genotypes at flowering stage was collected in eppendorf with help of Hamilton syringe. The fluid was collected during daylight between 04:00 to 06:00 pm to avoid merge of dew some time deposited on the leaves. Before collecting the droplets, leaf was trickled down into eppendorf from exuding sites. The amount of fluid exuded in volumetrically was determined through the help of Hamilton syringe and amount expressed in µl/tip. The nitrogen use efficiency was calculated as the ratio of the grain yield (kg grain $ha^{-1}$) to the N applied (kg N $ha^{-1}$). Grain yield from each plot was recorded and finally expressed as g $m^{-2}$. For calculation of biological yield, each plant was uprooted from ground level at maturity and then bundled and labeled and after drying, the weight of intact plant was determined before threshing and the total weight of the plant was recorded as biological yield in g $m^{-2}$. The correlation between guttation fluid, NUE, grain yield and biological yield vice versa was made. The data was analyzed statistically for calculating standard error of mean (SEm) and critical difference (CD) at 5% probability level (Panse and Shukhtme, 1978). The data shown in tables are the mean of two years for 2008 and 2009.

## RESULTS AND DISCUSSION

### Guttation fluid

The guttation fluid (GF) which oozed through the leaf tip of rice genotypes was directly affected with application of N fertilizer (Table 2). The amount of guttation fluid (GF) drastically increased from $N_0$ level to $N_{200}$ and maximum achieved at $N_{200}$ level of nitrogen applied. It is interesting to know that the applied nitrogen fertilizer exhibit positive correlation with guttation fluid (GF) for all rice genotypes. The maximum guttation fluid at flowering stage was observed for KRH-2 whiles the minimum for Kasturi followed by Tulsi, Vasumati and Kasturi. The reasons behind it, the KRH-2 is a hybrid rice genotype that has more capacity to extract available nitrogen from the soil and it showed better response to nitrogen fertilizer at different nitrogen levels. In addition to this, the hybrid and high yielding rice genotypes have more ability to guttate more amount of guttation fluid than inbred and low yielding rice varieties. Similar results were revealed by other researchers (Singh et al., 2008, 2009).

### Nitrogen use efficiency

Nitrogen use efficiency also showed a significant correlation among applied nitrogen fertilizer (Table 3) and also exhibited positive relationship with guttation fluid (Figure 1). The nitrogen use efficiency was increased with increasing N levels up to $N_{100}$ level (Table 3). Further increase in N levels decreased the NUE. It is due to that, at higher concentration of N the absorption exceeds the utilization. The KRH-2 was found most N efficient genotype whereas Kasturi least efficient. In field experiments, hybrid rice had a greater N efficiency than conventional rice (Lin et al., 1980). It was reported that NUE was increased from $N_{45}$ (20.00), up to $N_{90}$ (31.00) but further increase in N levels decreased nitrogen use efficiency as $N_{135}$ (29.00) and $N_{180}$ (19.00). Nitrogen use efficiency is a genotypic parameter and ranged from 35.6 to 51.6 (kg grain $kg^{-1}$ N absorbed) for different genotypes (Samonte et al., 2006). The current average nitrogen use efficiency (NUE) in the field is approximately 33% and substantial proportion of the remaining 67% is lost into the environment, especially

**Table 3.** Effect of N levels on NUE of five rice genotypes.

| Genotype | Nitrogen use efficiency (%) | | | | |
|---|---|---|---|---|---|
| | $N_0$ | $N_{50}$ | $N_{100}$ | $N_{200}$ | Mean |
| KRH-2 | 28.35 | 70.26 | 56.50 | 39.33 | 48.61 |
| Vasumati | 21.13 | 22.15 | 19.91 | 19.43 | 20.66 |
| Kasturi | 16.94 | 23.63 | 20.04 | 19.64 | 20.06 |
| Tulsi | 25.31 | 30.55 | 24.93 | 25.18 | 26.49 |
| Krishna Hamsa | 25.27 | 44.26 | 34.03 | 33.65 | 34.30 |
| Mean | 23.40 | 38.17 | 31.08 | 27.45 | |

| | Treatment (T) | Variety (V) | T × V |
|---|---|---|---|
| S.E.M. ± | 0.49 | 0.39 | 0.79 |
| CD ($p$ = 5%) | 1.70 | 1.14 | 2.29 |

**Table 4.** Effect of N levels on grain yield of five rice genotypes.

| Genotype | Grain Yield (g $m^{-2}$) | | | | |
|---|---|---|---|---|---|
| | $N_0$ | $N_{50}$ | $N_{100}$ | $N_{200}$ | Mean |
| KRH-2 | 311.00 | 759.33 | 768.00 | 610.00 | 612.08 |
| Vasumati | 259.00 | 291.67 | 300.00 | 296.67 | 286.84 |
| Kasturi | 229.67 | 341.67 | 283.33 | 300.67 | 288.84 |
| Tulsi | 301.00 | 383.33 | 383.33 | 408.33 | 369.00 |
| Krishna Hamsa | 301.00 | 583.33 | 508.33 | 541.67 | 483.58 |
| Mean | 280.33 | 471.87 | 448.60 | 431.47 | |

| | Treatment (T) | Variety (V) | T × V |
|---|---|---|---|
| S.E.M. ± | 24.09 | 21.71 | 43.56 |
| CD ($p$ = 5%) | 83.25 | 62.55 | 125.11 |

cropped areas (Abrol et al., 2007).

### Grain yield

The significant relationship between grain yield, applied nitrogen fertilizer and guttation fluid was recorded. The grain yield was found to be increased with increasing N doses and highest recorded at $N_{200}$ level for most of the genotypes. But for some rice genotypes like Kasturi (147.67) and Krishna Hamsa (123.67), it was highest at $N_{50}$ level than $N_{100}$ and for rice genotype Krishna Hamsa (131.00); it was maximum at $N_{50}$ levels than $N_{50}$ and $N_{100}$ levels (Table 4). The KRH-2 got maximum grain yield followed by Krishna Hamsa, Tulsi, Kasturi and Vasumati. This increased grain yield due to increases grain number in response to application of N fertilizers and enhanced availability of N. Besides this, number of grains per panicle was significantly influence by application of nitrogen fertilizer (Manzoor, et al., 2006; Zhenxie et al., 2008).

### Biological yield (g $m^{-2}$)

The biological yield is the result of several physiological as well as biochemical process and also depends on which types of genotype are used. It is noted that biological yield and guttation fluid showed significant relation with applied nitrogen fertilizer and vis-à-vis (Table 5 and Figure 1). The results of biological yield showed the similarity as the grain yield. It was utmost recorded for hybrid rice genotype KRH-2 and the least for rice genotype Tulsi (Table 5). Similar results were observed by Manzoor (2006). More straw yield could be a result of more biomass and it can be due to higher capability of hybrid rice to utilize more N through the expression of better growth by accumulating more dry matter (Meena et al., 2003).

### Relationship between GF, NUE, GY and BY

It was too interesting to observe that the yield related traits such as nitrogen use efficiency, grain yield and

**Table 5.** Effect of N levels on biological yield of five rice genotypes.

| Genotype | Biological yield (g m$^{-2}$) | | | | |
|---|---|---|---|---|---|
| | $N_0$ | $N_{50}$ | $N_{100}$ | $N_{200}$ | Mean |
| KRH-2 | 955.67 | 1757.67 | 2408.83 | 2402.33 | 1881.13 |
| Vasumati | 858.00 | 1341.52 | 1401.00 | 1596.83 | 1299.34 |
| Kasturi | 835.83 | 1526.80 | 1381.55 | 1441.83 | 1296.50 |
| Tulsi | 783.93 | 1283.48 | 1141.83 | 1295.50 | 1126.19 |
| Krishna Hamsa | 991.57 | 1851.00 | 1675.83 | 1876.23 | 1598.66 |
| Mean | 885.00 | 1552.09 | 1601.81 | 1722.54 | |

| | Treatment (T) | Variety (V) | T × V |
|---|---|---|---|
| S.E.M. ± | 33.28 | 37.20 | 74.62 |
| CD (*p* = 5%) | 115.03 | 107.16 | 214.32 |

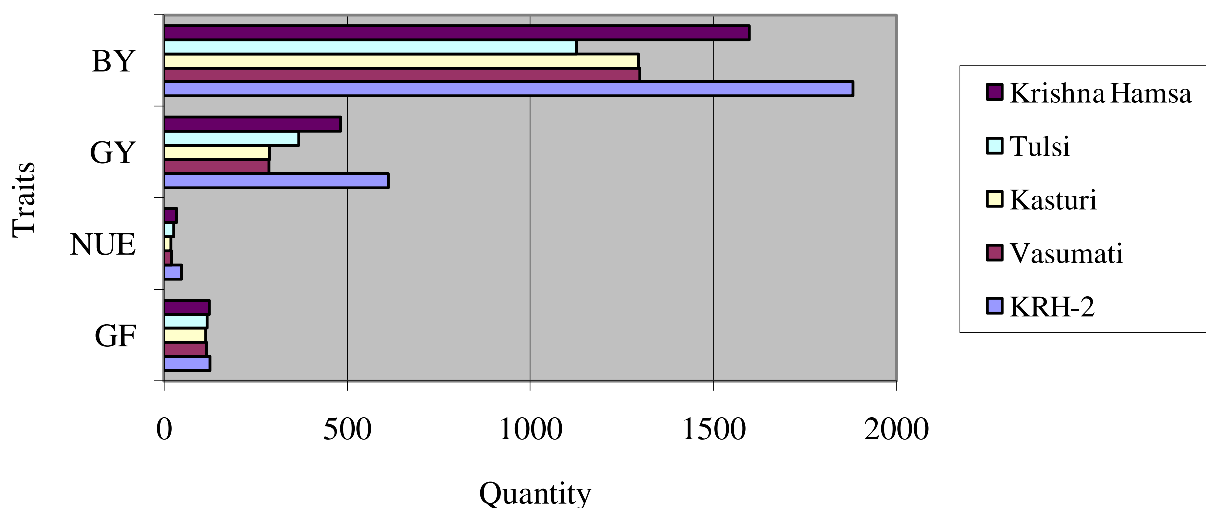


**Figure 1.** Relationship between Guttative Fluid-GF (µl/tip) and Nitrogen Use Efficiency-NUE (%), Grain Yield-GY (g m$^{-2}$), Biological Yield-BY (g m$^{-2}$) amongst five genotypes of rice.

biological yield illustrated positive correlation with guttation fluid and vice-versa. In addition to this, strong positive correlation was observed between guttation fluid (GF), nitrogen use efficiency (NUE), grain yield (GY) and biological yield (BL) amongst five rice genotypes treated with four nitrogen doses (Figure 1). The genotype showed greater amount of guttation fluid oozed through the leaf tip, same time got maximum NUE, grain yield and biological yield too. Out of five rice genotypes, the rice genotype KRH-2 gained more guttation fluid and NUE followed by Krishna Hamsa, Tulsi, Vasumati and Kasturi. The reasons behind it is that the KRH-2 is a hybrid nature genotype having better response to nitrogen fertilizer and also because of more capacity to extract N and guttate more amount of guttation fluid than inbred and low yielding rice varieties. It was interesting to know that these findings were supported by other researcher they observe that the higher the volume of exuded fluid by the varieties, the greater their panicle weights and grain yield (Singh et al., 2008, 2009).

## Conclusion

A positive correlation was concluded between guttation fluid (GF) and nitrogen use efficiency (NUE), grain yield (GY) and biological yield (BY) for all rice genotypes namely KRH-2, Kasturi, Krishna Hamsa, Tulsi and Vasumati treated with $N_0$, $N_{50}$, $N_{100}$ and $N_{200}$ kg ha$^{-1}$ levels. Besides this, based on the yield traits, it was also concluded that KRH-2 rice genotype revealed more efficiency to nitrogen uptake due to secretion of more guttation fluid. Further, guttation fluid can be used as a physiological marker for screening high yielding rice varieties for high nitrogen use efficiency. Secretion of guttation fluid by leaf tip is directly associated with better

uptake of nitrogen and nitrogen use efficiency and amount of guttation fluid enhanced by application of different levels of nitrogen fertilizer. Hence, this study offer good opportunity for rice researcher to improve rice yield through this way and mapping the genes controlling this trait and producing rice plant with increase guttation fluid at different N levels.

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*Full Length Research Paper*

# The reproducibility of random amplified polymorphic DNA (RAPD) profiles of *Streptococcus thermophilus* strains with XD9, M13 and OPI-02 MOD primers

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**We optimized the conditions for generating random amplified polymorphic DNA (RAPD) profiles of *Streptococcus thermophilus* strains by using the polymerase chain reaction (PCR). Several factors can cause the amplification of false and non reproducible bands in the RAPD profiles. We tested three primers, OPI-02 MOD, M13 and XD9 throughout this study. In addition, we tested different concentrations of primer, DNA template and Taq DNA polymerase. We adjusted the ratio of the primer to DNA template. All the three primers yielded reproducible profiles on several days, under optimized concentrations of components and cycling parameters used. The bands of such profiles probably corresponded to perfect annealing sites amplified with good efficacy or present in multiple copies in the genome. Five months later, repeated experiment generated identical bands. However, extra faint bands were detected with M13 and XD9 primers, possibly, corresponding to nonspecific binding resulting from slight variation in temperature or calibration of the thermocycler. Therefore, OPI-02 MOD was determined as the most reliable primer for reproducible profiles of *S. thermophilus* strains.**

**Key words:** *Streptococcus thermophilus*, random amplified polymorphic DNA (RAPD), DNA template, Taq DNA polymerase, OPI-02MOD, XD9, M13, optimization, reproducibility.

## INTRODUCTION

*Streptococcus thermophilus* is considered to be the second most important species of industrial lactic acid bacteria, after *Lactococcus lactis*, with a market value of about 40 billion US$; over $10^{21}$ live cells are ingested annually by the human population. It is well known as a starter culture component in yoghurt fermentation and cheese making (De Vuyst and Tsakalidou, 2008; Hols et al., 2005). Despite the fact that no natural habitat for this species outside the dairy environment has been identified, considerable inter-strain diversity has been observed (O'Sullivan and Fitzgerald, 1998).

Random amplified polymorphic DNA technique (RAPD) constitutes a useful technique for the study of genetic polymorphism of DNA. It involves the amplification of random segments of genomic DNA by polymerase chain reaction (PCR), using short single primers of arbitrary sequence (Williams et al., 1990).

This molecular approach has been widely utilized to identify and study the level of biodiversity among *S. thermophilus* strains (Morandi and Brasca, 2012; Lazzi et al., 2009; Rizzotti et al., 2009; Andrighetto et al., 2002; Mora et al., 2002; Giraffa et al., 2001; Moschetti et al., 1998). It is a very sensitive and simple technique and is suggested as a fast tool for characterization of a large

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number of lactic acid bacteria isolated from dairy products (Lazzi et al., 2009; Ramos et al., 2008); but, this method is prone to poor reproducibility in the band pattern, due to even small changes in reaction conditions; for this reason, it should be highly standardized to achieve satisfactory reproducibility (Skorić et al., 2012; Singh et al., 2009).

The PCR conditions for RAPD analysis can be optimized by varying the concentrations of the reaction mixture components and other reaction factors such as primer annealing, primer extension, denaturation time (Skorić et al., 2012; Singh et al., 2010; Fraga et al., 2005; Tyler et al., 1997; Wolff et al., 1993). The aim of this study was to optimize the concentrations of Taq DNA polymerase, DNA template and primer of PCR reactions and to study their effect on RAPD profiles and reproducibility. The RAPD-PCR of *S. thermophilus* geno-mic DNA was performed with XD9, M13 and OPI-02 MOD primers. It is useful to develop a reliable RAPD-PCR fingerprinting method for further study of *S. thermophilus* genetic diversity at strain level and to be able to distinguish between a large number of new isolates of *S. thermophilus* quickly and efficiently.

## MATERIALS AND METHODS

### *Streptococcus thermophilus* strains

Twenty one (21) *S. thermophilus* strains (S1-3, K1-15, N4-3, K1-31, N8-2, S1-3, K1-15, K1-7, N5-4, K1-1, S1-3, N2-1, K1-15, N8-2, N6-1, K1-22, N6-2, S1-1, K1-26, K1-12, N3-1) isolated from Turkish traditional yoghurts, were used in this study (from the collection of Food Analysis Laboratory, Middle East Technical University, Ankara, Turkey). The reference strain *S. thermophilus* LMG18311 and the *S. thermophilus* strain Yo-mix 410-3 (from Danisco Commercial starter culture) were also included. They were grown at 42°C in M17 broth, pH 6.8 and stored at -80°C in M17 glycerol.

### DNA isolation

The DNA of *S. thermophilus* strains was extracted by The Gene JET™ Genomic DNA Purification kit (Fermentas) according to the manufacturer's instructions. DNA concentration and the ratio A260/A280, for checking the purity of DNA, were calculated using UV Spectrophotometer (NanoDrop). All the DNA solutions obtained were stored at -20°C.

### Optimization of RAPD reaction

The following three primers, obtained from Metabion International (Deutschland) were used, separately, for amplification: XD9 primer: 5'-GAAGTCGTCC-3' (Moschetti et al., 1998) ; OPI-02 MOD primer: 5'-GCTCGGAGGAGAGG-3' (Mora et al., 2002) and M13 primer: 5'-GAGGGTGGCGGTTCT-3' (Huey and Hall, 1989).

PCR amplification was carried out in MJ mini personal Thermal cycler (Bio-Rad). The cycling programs used were those described by Moschetti et al. (1998), for XD9 primer and Mora et al. (2002) for OPI-02 MOD primer. The cycling program with M13 primer consisted of an initial denaturation step at 94°C for 2 min and then 40 cycles of 94°C for 1 min; 42°C for 20 s and 72°C for 2 min. The final elongation was performed at 72°C for 10 min. Amplification was performed in a final volume of 50 µl RAPD-PCR reaction mixture containing 1X PCR buffer. The optimization of RAPD-PCR reaction conditions was performed by varying concentrations as follows: 40, 60, 80, 100, 150 ng of DNA template, 0.5, 1, 1.25, 1.3, 1.5, 2, 2.5, 3.5 U of Taq DNA polymerase (Fermentas) and 0.1, 0.2, 0.3, 0.4, 0.5, 1 µM of primer.

The concentrations of $MgCl_2$ (3.5 mmol $l^{-1}$for XD9 primer, 2.5 mmol $l^{-1}$ for OPI-02 MOD primer and 3 mmol $l^{-1}$ for M13 primer) and dNTPs (200 µmol $l^{-1}$ of each dATP, dCTP, dGTP and dTTP) were maintained constant with values as given by Fermentas Manufacturer and the authors above. Reactions without DNA were used as negative controls, which were prepared for each set of reaction mixture and included in all gels.

RAPD products were resolved by electrophoresis at 90 V in 1.5% (w/v) in Basica LE Prona agarose in 1XTBE gels. 100 bp Plus DNA ladder (Fermentas) was used as a molecular size standard. Gels were stained in 0.5 mg of ethidium bromide and 500 ml of distilled water for 20 min in a covered container; they were destained in distilled water for 5 min and images were captured by TIFF files using the Gel Doc XR digital imaging system (Bio-Rad).

### Reproducibility study

DNA extraction and RAPD-PCR amplification were performed at least two times, on several days. The *S. thermophilus* strains used for the optimization were: Yo-mix 410-3, S1-3, K1-15, N4-3, K1-31 with XD9 primer, N8-2, S1-3, K1-15 with OPI-02 MOD primer and K1-7, N5-4, K1-1, S1-3, N2-1, K1-15, N8-2 with M13 primer. Then, the optimum components' concentrations of PCR reaction mixture obtained were applied for amplification of the following *S. thermophilus* strains: N6-1, LMG18311 (DNA extraction was performed three times for *S. thermophilus* LMG 18311), K1-22, N6-2, S1-1, K1-26, K1-12, N3-1. The experiment was repeated three times, with the three primers, on several days.

About five months after optimization experiments, the RAPD-PCR amplification was performed with the three primers again. Six strains were tested for each primer as follows: N6-1, LMG18311, K1-22, N6-2, S1-1, K1-26 with OPI-02MOD primer and XD9 primer. The amplification was carried out with *S. thermophilus* strains, LMG18311, K1-12, N3-1, K1-31, N4-3, Yo-mix 410-3, for M13 primer.

## RESULTS

### Optimization of RAPD protocol

In order to optimize RAPD-PCR method, for *S. thermophilus* strains, several concentrations of template DNA, Taq DNA polymerase and primers were tested. The concentrations of PCR mixture selected were optimized according to their relative effects on RAPD amplifications in terms of the highest number and intensity of bands, the generation of clear, scorable and reproducible amplified products.

The ratio of the primer concentration to DNA template concentration is one of the critical factors affecting RAPD profiles and reproducibility (Thangaraj et al., 2011; Tyler et al., 1997; Davin-Regli et al., 1995). The titer of the template DNA concentration should be carefully determined against a fixed primer concentration to obtain ideal conditions (Tyler et al., 1997).

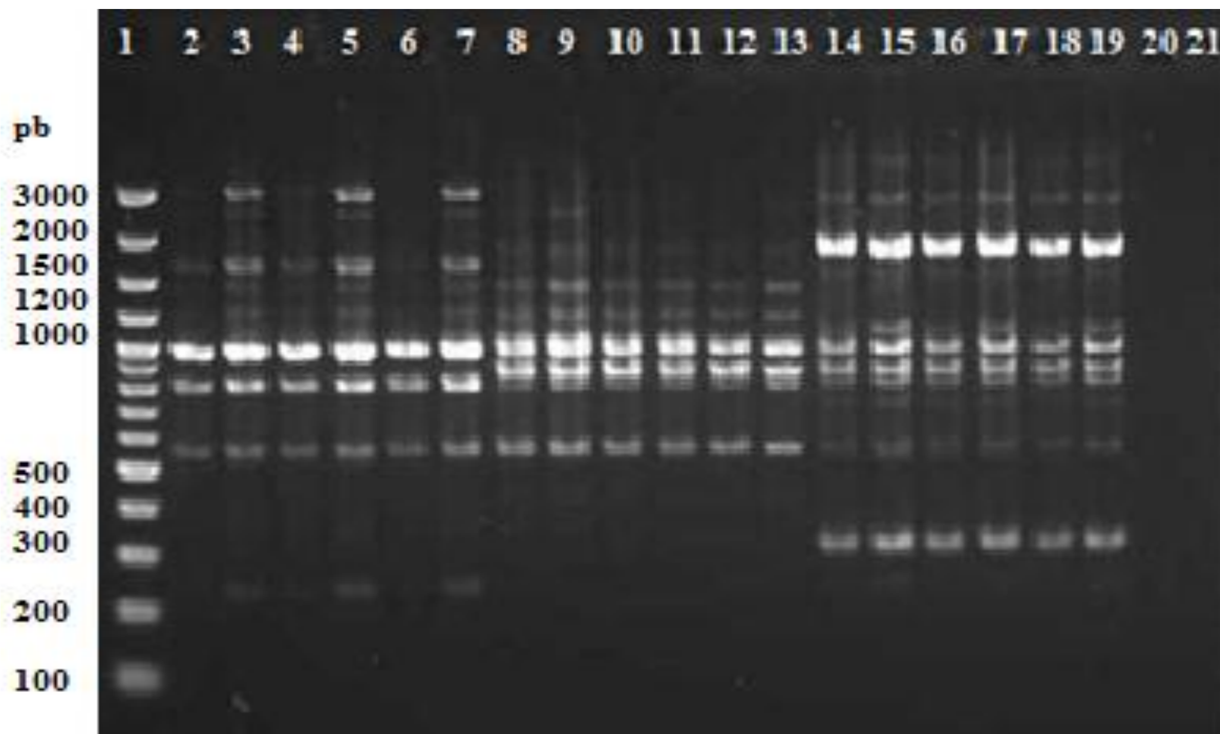


**Figure 1.** RAPD profiles of *S. thermophilus* strains with 1 µmol $l^{-1}$ of OPI-02 MOD primer; 1, Molecular size marker; 2, N8-2 strain, 80 ng of DNA template, 1.3 U of Taq DNA polymerase; 3, N8-2 strain, 80ng of DNA template, 2.5 U of Taq DNA polymerase; 4, N8-2 strain, 100 ng of DNA template, 1.3 U of Taq DNA polymerase; 5, N8-2 strain, 100 ng of DNA template, 2.5 U of Taq DNA polymerase; 6, N8-2 strain, 150 ng of DNA template, 1.3U of Taq DNA polymerase; 7, N8-2 strain, 150 ng of DNA template, 2.5 U of Taq DNA polymerase; 8, S1-3 strain, 80ng of DNA template, 1.3 U of Taq DNA polymerase; 9, S1-3 strain, 80 ng DNA template 2.5 U of Taq DNA polymerase; 10, S1-3 strain, 100 ng of DNA template, 1.3 U of Taq DNA polymerase; 11, S1-3 strain, 100 ng of DNA template, 2.5 U of Taq DNA polymerase; 12, S1-3 strain, 150 ng of DNA template, 1.3 U of Taq DNA polymerase; 13, S1-3 strain, 150 ng of DNA template, 2.5 U of Taq DNA polymerase; 14, K1-15 strain, 80 ng of DNA template, 1.3U of Taq DNA polymerase; 15, K1-15 strain, 80 ng of DNA template, 2.5 U of Taq DNA polymerase; 16, K1-15 strain, 100 ng of DNA template, 1.3 U of Taq DNA polymerase; 17, K1-15 strain, 100 ng of DNA template, 2.5 U of Taq DNA polymerase; 18, K1-15 strain, 150 ng of DNA template, 1.3U of Taq DNA polymerase; 19, K1-15 strain, 150 ng of DNA template, 2.5 U of Taq DNA polymerase; 20, negative control, 1.3 U of Taq DNA polymerase; 21, negative control, 2.5 U of Taq DNA polymerase.

It was observed that the use of 80, 100 and 150 ng of DNA template concentrations, with a fixed concentration (1 µmol $l^{-1}$) of OPI-02MOD primer provided the same result when using 1.3 or 2.5 U of Taq DNA polymerase (Figure 1). Similar RAPD profiles were obtained using 1.3 and 2.5 U of Taq DNA polymerase for S1-3 and K1-15 strains. However, there was an increase in the number of the detectable bands by increasing the concentrations of Taq DNA polymerase from 1.3 to 2.5 U for N8-2 strain. As a result, identical RAPD profiles were obtained using 2.5 and 3.5 U of Taq DNA polymerase with 100 ng of DNA template for the three strains (data not shown). Subsequently, the optimized PCR reaction mixture included: 1 µmol $l^{-1}$ OPI-02 MOD primer, 100 ng of DNA template, 2.5 U of Taq DNA polymerase, 1X PCR buffer, 2.5 mmol $l^{-1}$ $MgCl_2$, 200 µmol $l^{-1}$ of each of the four dNTPs in a final volume of 50 µl.

Identical profiles were generated, with a fixed concentration of 1 µmol $l^{-1}$ XD9 primer, using concentrations of 100 and150 ng DNA template and 2 U of Taq DNA polymerase for the five *S. thermophilus* strains tested (data not shown). Moreover, amplification of 100 ng of DNA template, using 1.5 and 2 U Taq DNA polymerase, produced identical patterns (Figure 2A and B). Subsequently, optimized RAPD-PCR reaction contained 50 µl; 100 ng of DNA template, 1 µmol $l^{-1}$ of XD9 primer, 2 U of Taq DNA polymerase, 1X PCR buffer, 3.5 mmol $l^{-1}$ $MgCl_2$, and 200 µmol $l^{-1}$ of each of the four dNTPs.

Amplification bands were observed in the negative controls with ratios of M13 primer to DNA template of 0.3 µmol $l^{-1}$ / 100 ng, 0.4 µmol $l^{-1}$ / 100 ng, 0.5 µmol $l^{-1}$ / 100 ng and 1 µmol $l^{-1}$ / 100 ng (Figures 3A, 4A and B). However, no bands were detected, in the negative control, with a ratio of 0.2 µmol $l^{-1}$ / 100 ng (Figure 3B). Similar RAPD profiles were obtained with ratios of M13 primer to template DNA of 0.2 µmol $l^{-1}$ / 100 ng, 0.3 µmol $l^{-1}$ / 100 ng and 0.4 µmol $l^{-1}$ / 100 ng (Figure 3A and B).

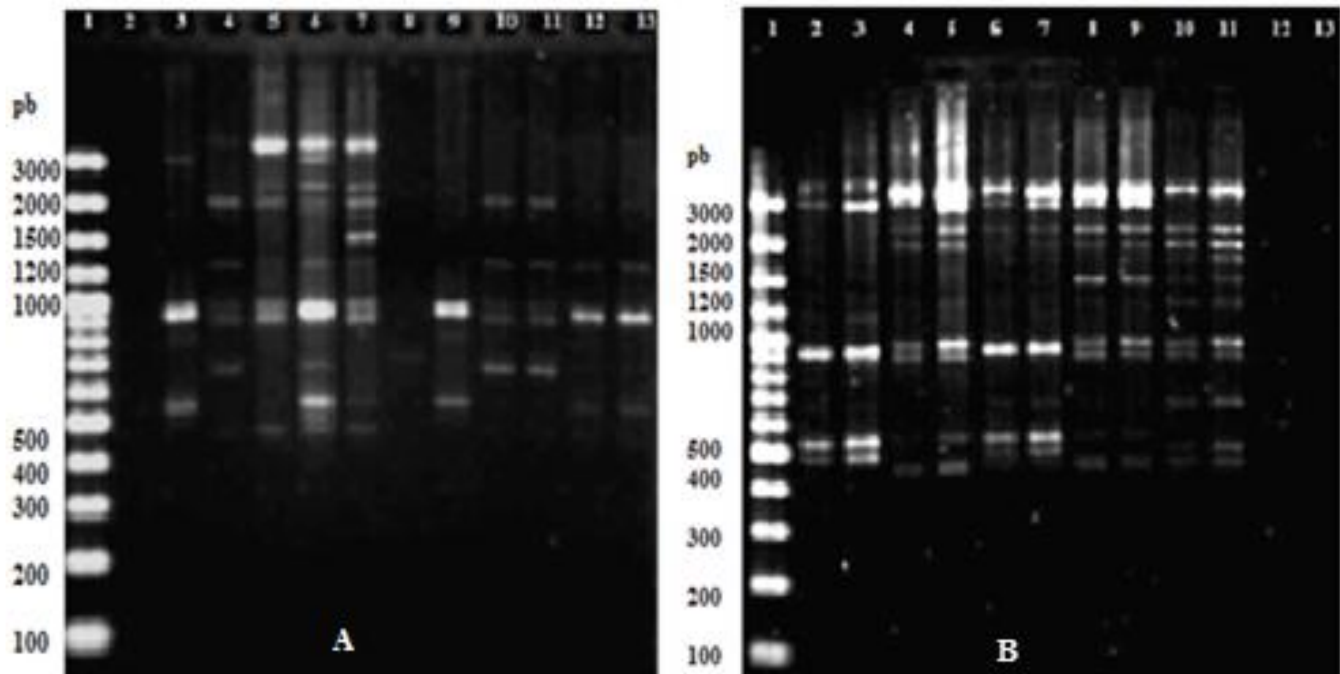


**Figure 2. (A)** RAPD profiles of *S. thermophilus* strains with 1 µmol $l^{-1}$ of XD9 primer, 100 ng of DNA template, 1.25 U of Taq DNA polymerase. 1, molecular size marker; 2, negative control; 3, 410-3 strain; 4, K1-31 strain; 5, S1-3 strain; 6, K1-15 strain; 7, N4-3 strain; 8, negative control; 9, 410-3 strain; 10, K1-31 strain; 11, K1-31 strain; 12, K1-15 strain; 13, K1-15 strain. **(B)** RAPD profiles of *Streptococcus thermophilus* strains with 1 µmol $l^{-1}$ of XD9 primer, 100 ng of DNA template; 1, molecular size marker; 2, 410-3 strain, 1.5 U of Taq DNA polymerase; 3, 410-3 strain, 2 U of Taq DNA polymerase; 4, S1-3 strain, 1.5 U of Taq DNA polymerase; 5, S1-3 strain, 2 U of Taq DNA polymerase; 6, K1-15 strain, 1.5 U of Taq DNA polymerase; 7, ) K1-15 strain, 2 U of Taq DNA polymerase; 8, N4-3 strain, 1.5 U of Taq DNA polymerase; 9, N4-3 strain, 2 U of Taq DNA polymerase; 10, K1-31 strain, 1.5 U of Taq DNA polymerase; 11, K1-31 strain, 2 U of Taq DNA polymerase; 12, negative control, 1.5 U of Taq DNA polymerase; 13, Negative control, 2 U of Taq DNA polymerase.

Therefore, 0.2 µmol $l^{-1}$ of M13 primer, 100 ng of bacterial DNA and 1 U *Taq* DNA polymerase were chosen as optimum conditions since no bands were detected in the negative control under these conditions (Figure 3B). Moreover, using 1 and 1.5 U of Taq DNA polymerase yielded identical patterns for all tested strains (result not shown). As a result, the optimized PCR reaction mixture included 100 ng of bacterial DNA, 1X PCR buffer, 3 mmol $l^{-1}$ $MgCl_2$, 200 µmol $l^{-1}$ of each of the four dNTPs, 0.2 µmol $l^{-1}$ of M13 primer and 1 U Taq DNA polymerase.

### The reproducibility of RAPD profiles

The reproducibility of band patterns generated using the optimized parameters was confirmed, in several days, for the three primers, M13, OPI-02 MOD, XD9.

Five months later, results of RAPD PCR amplification performed with the same three primers were compared with those already obtained. Six strains tested produced identical patterns with OPI-02MOD primer, while four from the six strains produced identical patterns with M13 primer and only one was identical with XD9 primer. PAPD profiles of N4-3 strain and Yo-mix 410-3 strain showed two extra faint bands in their profiles, with M13 primer as compared to those obtained in the former five months. RAPD profiles of LMG 18311, Yo-mix 410-3, N6-2, K1-26 and N6-1 strains showed one, three, one, two and four extra faint bands, respectively with XD9 primer (M13 primer; Figure 5).

## DISCUSSION

The different concentrations of the RADP mixture tested had different degrees of influence on the RAPD patterns and their reproducibility. An efficient protocol for RAPD analysis should be reasonably resistant to variations in template DNA concentrations (Skorić et al., 2012).

In our experiments, identical profiles were reached over a range of different concentrations of template DNA. Similar observations have been reported by Skorić et al. (2012) and Wolff et al. (1993). For instance, chrysanthemum DNA amplification was relatively constant between a large range of 1 and 500 ng template DNA (Wolff et al., 1993). With regard to the Taq polymerase concentration, a threshold concentration, in which the RAPD profiles were identical, was noticed; same result has been re-

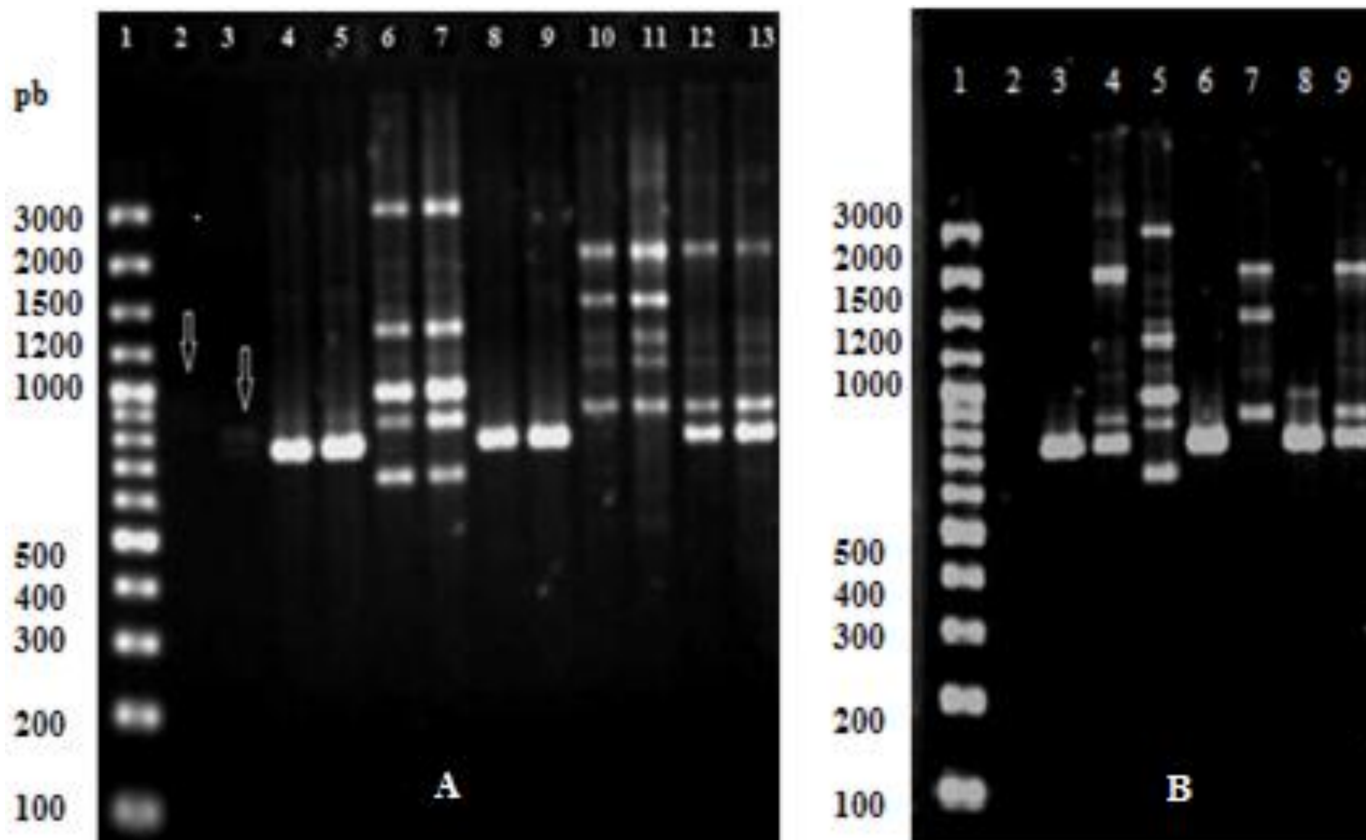


**Figure 3. (A)** RAPD profiles of *S. thermophilus* strains with M13 primer, 100 ng of DNA template, 1 U of Taq DNA polymerase. 1, Molecular size marker; 2, negative control, 0.3 $\mu$mol $l^{-1}$ of primer; 3, negative control, 0.4 $\mu$mol $l^{-1}$ of primer; 4, K1-7 strain, 0.3 $\mu$mol $l^{-1}$ of primer; 5, K1-7 strain,0.4 $\mu$mol $l^{-1}$ of primer; 6, N5-4 strain, 0.3 $\mu$mol $l^{-1}$ of primer; 7, N5-4 strain, 0.4 $\mu$mol $l^{-1}$ of primer; 8, K1-1 strain, 0.3 $\mu$mol $l^{-1}$ of primer; 9, K1-1 strain, 0.4 $\mu$mol $l^{-1}$ of primer; 10, S1-3 strain, 0.3 $\mu$mol $l^{-1}$ of primer; 11, S1-3 strain, 0.4 $\mu$mol $l^{-1}$ of primer; 12, N2-1 strain, 0.3 $\mu$mol $l^{-1}$ of primer; 13, N2-1 strain, 0.4 $\mu$mol $l^{-1}$ of primer. **(B)** RAPD profiles of *S. thermophilus* strains with M13 primer, 100 ng of DNA template, 1 U of Taq DNA polymerase, 0.2 $\mu$mol $l^{-1}$ of primer. 1, Molecular size marker; 2, negative control; 3, K1-7 strain; 4, N2-1 strain; 5, N5-4 strain; 6, K1-1 strain; 7, S1-3 strain; 8, K1-15 strain; 9, N8-2 strain.

ported (Skorić et al., 2012; Fraga et al., 2005). When using lower concentrations of template DNA or Taq DNA polymerase, no visible amplification or lower number and intensity of bands were detected (results not shown).

In this work, the effect of primer concentration on the obtained results was noticed, particularly with M13 primer; whereas, identical profiles were observed using a range of ratios of primer to DNA template from 0.2 to 0.4 µmol $l^{-1}$ / 100 ng. At lower ratio of 0.1 µmol $l^{-1}$ / 100 ng, different RAPD profiles were observed for N5-4, S1-3 and N2-1 strains (result not shown). In addition, at higher ratio of M13 primer to DNA template of 0.5 and of 1 µmol $l^{-1}$ / 100, extra bands were generated. This was clear when comparing profiles of N8-2 strain in Figures 3B (lane 9), 4A (lane9) and 4B (lane 5). The same observation was demonstrated with profiles of K1-15 strain in Figures 3B (lane 8), 4A (lane 13) and 4B (lane 7). At higher ratio of primer/template, rare or inaccessible sites can be amplified (Davin-Regli et al., 1995). In this case, bands could also result from the increase in weaker mismatch annealing of the primer to the target (Tyler et al., 1997; Caetano-Anolles et al., 1992). In addition, at higher ratio of primer/DNA template, the amplification of artefactual bands in the control samples without DNA has also been mentioned (William et al., 1990). This phenomenon was observed in the results with ratios of M13 primer to DNA template of 0.3 µmol $l^{-1}$ / 100 ng, 0.4 µmol $l^{-1}$ / 100 ng, 0.5 µmol $l^{-1}$ / 100 ng and 1 µmol $l^{-1}$ / 100 ng (Figure 3A, 4A and 4B). This can be explained by the possible contaminants or nonspecific products (primer-dimers) (Singh et al., 2010; Harini et al., 2008; Padmalatha and Prasad, 2006; Raghunathachari et al., 2000; Pan et al., 1997). The first possibility was excluded since it was checked by repeating our experiment with new reagents. Therefore, the second possibility is the most probable. Moreover, the artefactual bands in the negative control and extra bands (of N8-2 and K1-15 strains) increased by raising the concentration of M13 primer.

Therefore, the extra bands of N8-2 K1-15 strains generated with high M13 primer concentration could not

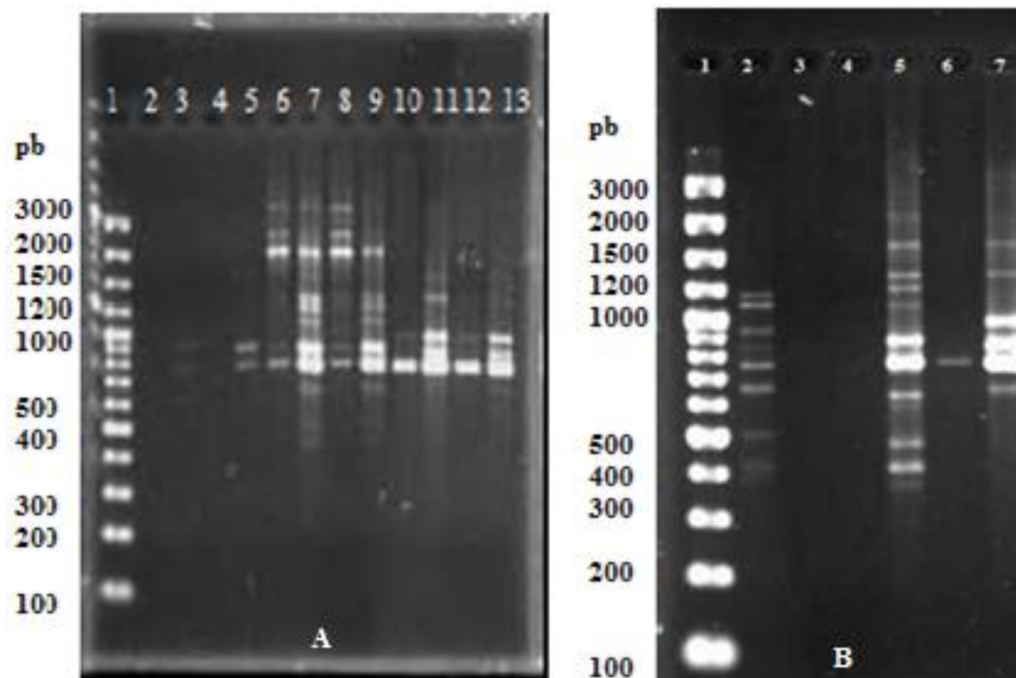


**Figure 4. (A)** RAPD profiles of *S. thermophilus* strains with 0.5 µmol l-1 of M13 primer. (5) Negative control; (9), N8-2 strain; (13), K1-15 strain. **(B)** RAPD profiles of *S. thermophilus* strains with 1 µmol l-1 of M13 primer. (2), negative control; (5), N8-2 strain; (7), K1-15 strain.

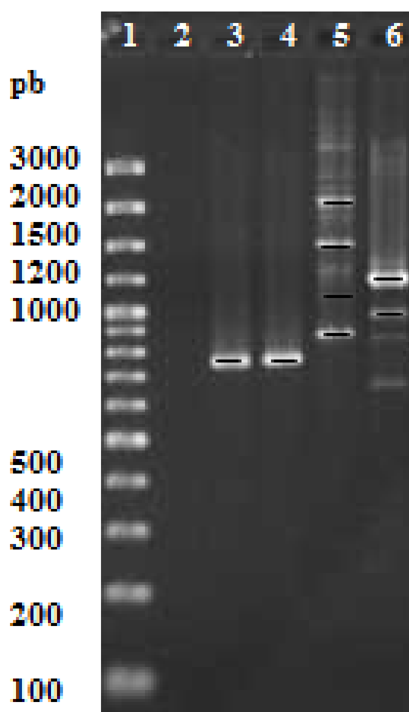


**Figure 5.** RAPD profiles of *S. thermophilus* strains with M13 primer was generated five months later. The black lines indicate the reproducible bands detected five months before. We can see the extra faint bands of strains (5), N4-3 and (6), Yo-mix 410- 3 without black lines.

be only due to the amplification of rare or inaccessible sites on DNA. The primer-dimers phenomenon could also affect RAPD strain's profiles. These nonspecific products could be amplified in the presence of a DNA template, as has been already reported by Pan et al. (1997).

The optimized conditions led to RAPD pattern's bands reproducibility with OPI-02MOD, M13, XD9 primers. Such bands correspond to perfect annealing sites amplified with good efficacy or present in multiple copies in the genome (Davin-Regli et al., 1995). When the experiments were repeated five months later, identical strains patterns were generated with OPI-02 MOD. However, the reproducible bands persisted in the profiles that have resulted from amplification with XD9 and M13 primers with the appearance of extra faint bands. This cannot be due to the contamination or degradation of stocked DNA over time (Black et al., 1992). Whereas, the five month's DNA samples gave identical patterns with OPI-02 MOD, contrary to XD9 primer. Moreover, the amplification of new extracted DNA of *S. thermophilus* LMG 18311, with XD9 primer, generated an extra faint band. However, it yielded identical profiles with OPI-02 MOD and M13 primers.

The study by Saunders et al. (2001) demonstrated that thermal cycler calibration and temperature monitoring have an important effect on RAPD profiles reliability and repeatability. The extra faint bands obtained could be a consequence of slight variation in annealing temperature or calibration of the thermocycler generating the amplifi-

cation of nonspecific DNA target sites.

In conclusion, OPI-02 MOD primer seemed to be the most reproducible, followed by M13 primer. XD9 primer showed the worst performance. Authors noted that, some primers are extremely reliable whereas others gave notoriously inconsistent results (Tyler et al., 1997; Grosberg et al., 1996; Bielawski et al., 1995; Penner et al., 1993). This could be explained by the presence of high amount of specific target sequence for OPI-02 MOD primer on *S. thermophilus* DNA template as compared to nonspecific sequences, and this could have a major effect on its good reproducibility.

One of the purposes of optimizing RAPD-PCR conditions is to increase the specificity and efficiency of primer-template interactions. Higher number of specific sites than nonspecific sites should be reasonably in favor of primer/specific target site interaction. Subsequently, varying slightly the temperature or calibration of thermocycler causes RAPD-PCR intralaboratory or interlaboratory non reproducibility. However, such slight variations may not have a significant consequence on the reproducibility for such primer/DNA template (OPI-02 MOD/ *Streptococcus thermophilus* DNA). Ramos et al. (2008) concluded that, at least in part, problems of repeatability attributed to RAPD markers could be due to bias in the selection of loci and primers and not necessarily the RAPD technique per se.

The reproducibility problem of RAPD-PCR can be overcome, in principle, by using rigid laboratory protocols and doing repeatability tests. The purpose of such tests is to repeat the analyses and retain only the bands that appear in both initial and later screenings for further analyses (Telles and Soares, 2007; Santos et al., 2007). Ramos et al. (2008) developed a procedure to optimize repeatability and avoid bias in sampling loci for genetic analyses based on RAPD data.

Using such reproducible OPI-02 MOD primer in studying *S. thermophilus* genetic diversity, at different laboratories or at different time periods within the same laboratories can be efficient. The strict standardization of RAPD-PCR conditions has to be applied using the same brand of Taq DNA polymerase, same model of Thermocycler, controlling the temperature and calibration of the Thermocycler.

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*Full Length Research Paper*

# Genetic structure of populations of *Mugil cephalus* using RAPD markers

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**Genetic structure of four populations of *Mugil cephalus* from Gujarat, Maharashtra, Andhra Pradesh and Tamil Nadu in India was studied using randomly amplified polymorphic DNA (RAPD) markers. Five selective primers provided distinct and consistent RAPD profiles in all the four populations. The bands in the range 400 to 1200 bp were scored for consistent results. The RAPD profiles generated by all the five primers revealed varying degrees of polymorphism, ranging from 50.76 (primer E03) to 72.41% (primer E05). Nei's genetic diversity (h) among the four populations varied from 0.3717 ± 0.1460 (Gujarat population) to 0.5316 ± 0.1720 (Maharashtra population). Nie's highest genetic distance (0.8556) was observed between Tamil Nadu and Gujarat populations.**

**Key words:** *Mugil cephalus*, randomly amplified polymorphic DNA (RAPD), genetic structure, India.

## INTRODUCTION

Information on the genetic structure of fish is useful for optimizing identification of stocks, stock enhancement, breeding programs, management of sustainable yield and preservation genetic diversity (Dinesh et al., 1993; Gracia and Benzie, 1995; Tassanakajon et al., 1997, 1998). DNA polymorphisms have been extensively employed as a means of assessing genetic diversity in aquatic organisms (Ali et al., 2004). Randomly amplified polymorphic DNA (RAPD) fingerprinting offers a rapid and efficient method for generating a new series of DNA markers in fishes (Foo et al., 1995).

RAPD analysis is a technique based on the polymerase chain reaction (PCR) amplification of discrete regions of genome with short oligonucleotide primers of arbitrary sequence (Welsh and McClelland, 1990; Williams et al., 1990). This method is simple and quick to perform and most importantly, no prior knowledge of the genetic make-up of the organism is required (Hadrys et al., 1992). This technique has been used extensively to detect genetic diversity in plants (Williams et al., 1993), animals (Cushwa and Medrano, 1996) and microbes (Carretto and Marone, 1995). It has also been used to evaluate genetic diversity in various fish species such as tilapia (Naish et al., 1995), striped bass (Bielawski and Pumo, 1997), grouper (Asensio et al., 2002) and murrel (Nagarajan et al., 2006). *Clarias batrachus* (Garg et al., 2010), *Eutropiichthys vacha* (Chandra et al., 2010) and *Plecropomus maculates* respectively. The striped mullet, *Mugil cephalus* is the most widely distributed and of aquaculture importance among mullets. It is euryhaline and also fairly resistant to changing temperature (Chondar, 1999). This species is one of the most popular warm water fishes being cultured in tropical and sub-tropical regions (Pillai et al., 1984). Indian aquaculture is

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**Abbreviations: RAPD,** Random amplified polymorphic DNA; **PCR,** polymerase chain reaction; **EDTA,** ethylenediaminetetra acetate; **SDS,** sodium dodecyl sulfate.

**Table 1.** *Mugil cephalus* RAPD profiles obtained by five random primers.

| Primer | Number of band | Band size (bp) | Total DNA band | Polymorphic DNA band | Percentage polymorphic DNA band |
|---|---|---|---|---|---|
| E02 (5'GGTGCGGGAA3') | 1 - 4 | 512 - 930 | 38 | 22 | 57.89 |
| E03 (5'CCAGATGCAC3') | 3 - 6 | 548 - 1200 | 65 | 33 | 50.76 |
| E04 (5'GTGACATGCC3') | 1 - 3 | 416 - 822 | 60 | 39 | 65.00 |
| E05 (5'TCAGGGAGGT3') | 1 - 3 | 746 - 884 | 29 | 21 | 72.41 |
| E06 (5'AAGGCCCCTC3') | 1 - 4 | 522 - 1196 | 40 | 27 | 67.50 |

mainly restricted to carps and shrimps. To achieve higher aquaculture production, species diversification must be prioritized. *M. cephalus* is one of the candidate species for diversification in the aquaculture sector due to its euryhaline nature easy availability of seeds along the coasts. Therefore, studying genetic variation in *M. cephalus* could provide base line data for identifying stock with superior traits for breeding programs and also to formulate management strategies for sustainable utilization of the species. Despite its aquaculture importance, there is no information available on genetic structure of this species. Hence, the present study was carried out to ascertain the genetic stock structure of *M. cephalus* populations using versatile RAPD markers.

## MATERIALS AND METHODS

### Experimental animal

Specimens *M. cephalus* (n=200; 50 from each location) were collected from four different locations in India: Navsari, Gujarat (20.5800°N, 72.5500°E); Ratnagiri, Maharashtra (16.9800°N, 73.3000°E); Kakinada, Andhra Pradesh (16.9300°N, 82.2230°E) and Chennai, Tamil Nadu (13.0810°N, 80.2740°E). The muscle tissue was collected and preserved in 95% ethanol, and transported in ice to the lab. The samples were stored at -20°C until DNA was extracted.

### Extraction of genomic DNA

Total genomic DNA was isolated from muscle tissue according to DNA extraction method of Williams et al. (1990). Tissue (150 to 200 mg) was cut into smaller pieces in the presence of 1 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM ethylenediaminetetra acetate (EDTA), 100 mM NaCl) and transferred to a 2 ml Eppendorf tube. Then, proteinase K (300 µg/ml), sucrose (1%), and sodium dodecyl sulfate (SDS) (2%) were added to the tube. After incubation at 60°C, the lysate was extracted with phenol and chloroform/isoamyl alcohol. The DNA was precipitated with isopropanol and pellet was washed with 70% ethyl alcohol, dried, suspended in TE buffer (50 mM Tris-HCl, 10 mM EDTA). DNA quality and quantity were determined by 1.0% agarose gel electrophoresis and biophotometer (Eppendorf, Germany).

### RAPD-PCR amplification and product analysis

Five random primers (E02 to E06; Operon, USA) were screened based on the presence of intense, well distinguished and reproducible bands for further analysis. PCR reactions were performed in 25 µl volume containing 200 µmol/l each dNTP, 2 mmol/l $MgCl_2$, 1 x standard *Taq* polymerase buffer, 0.2 µmol/l random primer, 40 ng genomic DNA, and 0.75 U *Taq* polymerase. PCR reactions were carried out with initial denaturation of 4 min at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, 45 s annealing at 36°C, 2 min extension at 72°C, and one 8 min cycle at 72°C for final extension. Amplified products were separated on 1.5% agarose gel stained with ethidium bromide, run in 1 X TAE buffer at a constant 80 V (Sambrook and Russell 2001). The gels were imaged using a Syngene gel documentation system (USA).

### Data analysis

Only the reproducible and intense bands ranging from 400 to 1200 bp were scored to maintain the consistency across the samples of different populations. Bands observed in each lane were compared with all the other lanes of the same gel and reproducible bands were scored as present (1) or absent (0). Fragment sizes were estimated based on the 100 bp Plus DNA Ladder (Bangalore Genie, India) according to the algorithm provided in the Gene Tools Software. Data was analyzed using the POPGENE version 1.31 software (Yeh et al., 1999). It was also used to construct dendrograms based on genetic distances (Nei, 1972; Sneath and Sokal, 1973; Reynolds et al., 1983). The robustness of the dendrogram was tested using 1000 bootstraps.

## RESULTS AND DISCUSSION

The RAPD profiles of different populations from Navsari (Gujarat), Ratnagiri (Maharashtra), Kakinada (Andhra Pradesh) and Chennai (Tamil Nadu) were generated for four geographically different populations of *M. cephalus*. The RAPD fingerprints of a total of 200 individuals of *M. cephalus* were carried out using optimized RAPD-PCR conditions for five selected primers. The polymorphism pattern obtained for four populations is shown in Table 1.

All the selected five primers produced distinct and consistent RAPD profiles for *M. cephalus* from all the four populations (Figures 1 and 2). The primers generated bands in the range of 200 to 2,200 bp. However, only the repeatable major bands ranging from 400 to 1200 bp were scored for consistency. A total of 142 reproducible bands were obtained in the three populations for the five primers (Table 1). Generally, the number and size of the bands generated strictly depend upon the nucleotide sequence of the primer used and the source of the template DNA; resulting in the genome-specific finger-prints of random DNA bands (Welsh et al., 1991).

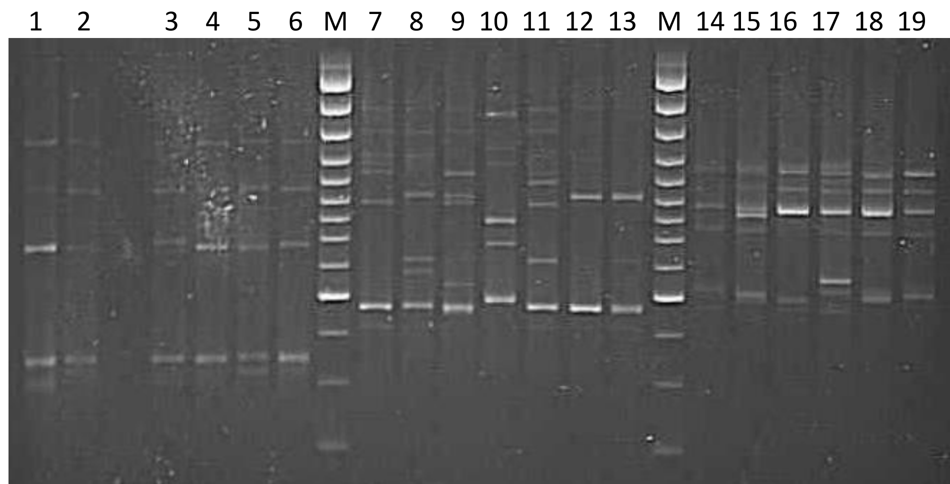


**Figure 1.** RAPD amplification of primer E03 in *M. cephalus*. Lanes 1-6, Samples from Gujarat; lanes 7-13, Maharashtra; lanes 14-19, Andhra Pradesh; M, molecular maker (100 bp plus DNA ladder).

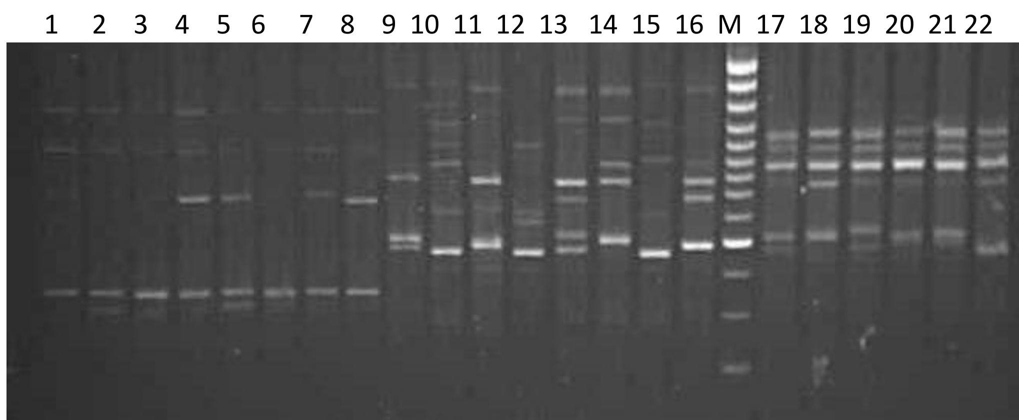


**Figure 2.** RAPD amplification of primer E04 in *M. cephalus.* Lane 1-8 samples from Gujarat; lanes 9-16, Maharashtra; lanes 17-22, Tamil Nadu; M, molecular maker (100 bp plus DNA ladder).

**Table 2.** Genetic diversity within four populations of *Mugil cephalus*.

| Population | Polymorphic loci (%) | Average genetic diversity |
|---|---|---|
| Gujarat | 88 | 0.3717 ± 0.1460 |
| Maharashtra | 76 | 0.5316 ± 0.1720 |
| Andhra Pradesh | 83 | 0.4419 ± 0. 2112 |
| Tamil Nadu | 70 | 0.4012 ± 0.1310 |

The RAPD profiles generated by all the five primers revealed varying degrees of polymorphism, ranging from 50.76% (primer E03) to 72.00% (primer E05). The range of number of bands and band size were 1 - 6 and 416 - 1196 bp, respectively.

The present study revealed a wide variation of polymorphic loci (70 - 88%) among the four populations. The highest level of polymorphism (88%) was exhibited by the Gujarat population whereas the lowest level of polymorphism (70%) was exhibited by the Tamil Nadu population. Nei's (1973) genetic diversity (h) among the four populations varied from 0.3717 ± 0.1460 (Gujarat population) to 0.5316 ± 0.1780 (Maharashtra population) (Table 2). Interestingly, two population specific bands

**Table 3.** Nie's genetic identity (above diagonal) and distance (below diagonal).

| Population | Gujarat | Maharashtra | Andhra Pradesh | Tamil Nadu |
|---|---|---|---|---|
| Gujarat | *** | 0.9214 | 0.1959 | 0.1544 |
| Maharashtra | 0.0886 | *** | 0.2086 | 0.1974 |
| Andhra Pradesh | 0.8241 | 0.8014 | *** | 0.9010 |
| Tamil Nadu | 0.8556 | 0.8126 | 0.1091 | *** |

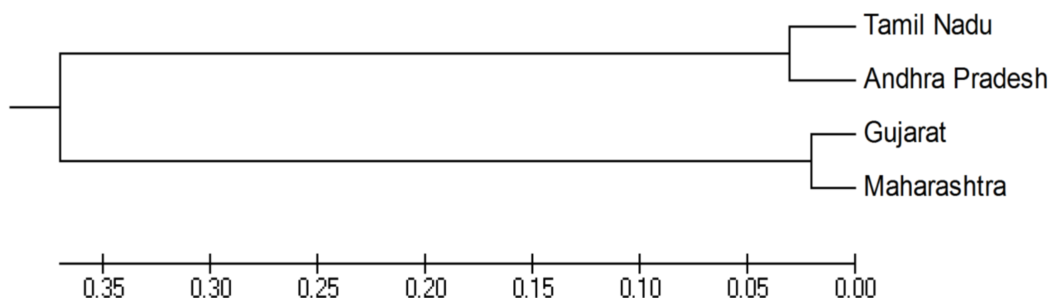


**Figure 3.** UPGMA dendrogram using Nei's unbiased genetic distance.

were found in the population of Andhra Pradesh (350 bp in E06 primer) and Gujarat (1000 bp in E04 primer). These population-specific unique bands can be used to detect any possible mixing of these populations, especially during selective breeding programmes (Ferguson et al., 1995). Tassanakajon et al. (1998), Mishra et al. (2009), Nagarajan et al. (2006) and Lakra et al. (2010) and Saad et al. (2012) also observed population specific bands in *Penaeus monodon Metapenaeus dobsonii Chaanna punctatus* and *Monoporeia affinis*, *Plectropomus leopardus* respectively.

Estimates of Nei's (1978) genetic distance demonstrated sufficient genetic divergence to discriminate the samples of different populations of *M. cephalus* (Table 3). The highest genetic identity (0.9214) and genetic distance (0.8556) was observed between the populations of Gujarat and Maharashtra and Tamil Nadu and Gujarat, respectively. A dendrogram based on Nei's genetic distance is shown in Figure 3. Two separate clades were identified on the dendrogram with the Maharashtra and Gujarat populations appearing one cluster, while the Tamil Nadu and Andhra Pradesh populations formed the other clade.

In conclusion, genetic stock structure of *M. cephalus* identified in this study using RAPD primers will be helpful in developing superior strain for aquaculture practices through selective breeding and formulating stock specific management measures for conservation and sustainable utilization of the species.

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African Journal of Biotechnology

*Full Length Research Paper*

# Evaluation of rough lemon (*Citrus jambhiri* Lush.) as rootstock for salinity tolerance at seedling stage under *in vitro* conditions

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***In vitro* approach was adopted to study the effect of salinity on survival and growth of rough lemon (*Citrus jambhiri* Lush.*)* seeds. North-West (Punjab) part of India has been facing a major problem of soil salinity for citrus orchards. Therefore, it is logical to study the salinity tolerance of common citrus rootstock, rough lemon (*Citrus jambhiri* Lush.), grown in the region. The seeds were treated with nine different doses of sodium salt. In all the treatments, leaves of rough lemon seedlings showed severe injury symptoms of chlorosis and necrosis while the seeds cultured in control did not show any injury. There was a significant decrease in seed germination, seedling height, internodal length, and subsequently plant weight with increasing concentration of salt. In contrast to the above characteristics, the length of primary roots increased proportionally with the increase in salt concentration in the culture media. As under stress conditions, the *in vitro* grown seedlings tend to increase the root length for its survival. In comparison to the control, salt treatments showed increased level of $Na^+$ and $Cl^-$ ions in the seedlings and also resulted in a decrease of $K^+/Na^+$ ratio. Tolerance index was found minimum (100) in control and maximum in 154mM NaCl treatments after 4 and 8 weeks.**

**Key words:** Citrus, salt, sensitivity, chlorosis, sodium chloride.

## INTRODUCTION

Around the world, citrus is one of the major horticultural crops and is relatively salt sensitive. In India, citrus industry is a third largest fruit industry after mango and banana with an area of 0.99 million ha area with an annual production of 96.4 x $10^8$ kg (CSO, 2007). In Punjab state, citrus crop covers an area of 44724 ha with an annual production of 9 x $10^8$ kg (DHP, 2011). Due to the negative influence on the yields of many crops, salinity is a major problem for citrus crop. In citrus, it has an adverse effect on tree growth and causes many physiological disorders. Primarily, salt-stress lowers the net $CO_2$ assimilation, stomata conductance, as well as water potential of citrus tree leaves in addition to the accumulation of excessive concentration of chloride or sodium in leaves (Al-Yassin, 2004). Excessive salts in soils interfere generally in plant metabolism and causes disturbance in water relations (Xoing and Zhu, 2002). In Southwestern region of Punjab, salinity is the major

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**Abbreviations: WS,** Plants without symptoms; **VMC,** very mild chlorosis; **PMC,** partial mild chlorosis; **BMA,** mild chlorosis to burns on the margins and apices of the lower leaves; **SDD,** intense damage with leaf loss followed by plant death; **NG,** no germination.

abiotic stress threatening citrus industry. Salt stress inhibits plant growth and reduces plant production due to water scarcity, ionic toxicity, and nutritional imbalance resulting in major yield loss.

Along with osmotic effects of soil salinity, Na and Cl uptake and their accumulation in plant tissues result in specific ionic toxicities. Competitive interactions among toxic ions (Na and Cl) and nutrients in the soil could induce nutrient imbalance and deficiencies (Grattan and Grieve, 1998). Numerous studies have shown that chloride tends to accumulate in citrus leaves. However, this accumulation may also be function of the age, position, and genotype of the shoots (Chapman, 1968; Walker et al., 1982; Banuls et al., 1997) which implicates the factors other than the root system in salt tolerance. Thus, $Cl^-$ resistance was affected not only by the root system, but also by shoot properties. Increased concentration of NaCl in the nutrition solution reduced growth proportionally and altered leaf and root mineral concentrations of all citrus rootstocks. The shoot height, leaf number, fresh weights of the seedlings, relative chlorophyll contents, chlorophyll fluorescence yields (Fv/Fm), net photosynthetic, and respiration rates in the leaves decreased with the increase in salinity level in the irrigation water (Zekri and Parsons, 1992; Anjum, 2008). Assessment of nutritional behavior of the new citrus rootstocks, *Forner-Alcaide no.5* (FA-5) and *Forner-Alcaide no.13* (FA-13), under saline conditions revealed that the accumulation of saline ions inhibits growth and nutrient uptake by citrus plants as elevated salt levels in the growth medium reduced the absorption of the mineral elements in all scion-rootstock combinations (Ginner et al., 2011). This problem can be prevented by soil and water management and/or using tolerant genotypes (Ashraf and Ahmad, 2000). In some glycophytes, the osmotic adjustment is related to the accumulation of proline, glycine-betaine, soluble sugars, and salt stress proteins (Ashraf and Harris, 2004), which results in adaptation by the plant to the saline environment (Munns et al., 2006). Apart from soil and water management, *in vitro* approaches have been adopted as one of the options to overcome salinity problems, under the laboratory conditions. In one such study, the plantlets were regenerated from the selected salt tolerant cell line of shamouti orange (*Citrus sinensis* L. Osbeck) and therefore, salt tolerance on the whole plant level was achieved (Ben-Hayyim and Goffer, 1989). Salt tolerant somaclonal variants in rice and *Poncirus trifoliate* through *in vitro* selection have been developed successfully (Beloualy and Bouharmont, 1992; Dang and Nuguyen, 2003)

Thus, keeping in view the salinity problems being faced in citrus plantations, the present investigation was conducted in rough lemon (*Citrus jambhiri* Lush), a major citrus rootstock in India and around the world, to find out a threshold value of salt tolerance and its influence on the growth and survival of *in vitro* grown seedlings.

## MATERIALS AND METHODS

### Growing media and treatments

The study was conducted in the tissue culture laboratory of Fruit Science Department, Punjab Agricultural University, Ludhiana. Seeds of rough lemon (*Citrus jambhiri* Lush.) extracted from healthy fruits, collected from the orchard of Punjab Agricultural University, Ludhiana, were surface sterilized with 0.1% mercuric chloride (w/v) for 3 min followed by washing with sterile distilled water twice. The sterilized seeds (20 for each treatment and 80 in total) were cultured on Murashige Skoog basal medium (Murashige and Skoog, 1962) consisting of MS salt solution supplemented with 30 g/l of sucrose, 7.5 g/l of agar and different concentrations of salt (NaCl) ranging from 0.1 to 0.9% and a control where no salt was added. One seed per test tube was cultured for every treatment. The pH of the medium was adjusted to 5.8 by adding 1N NaOH or 1N HCl solution drop wise before autoclaving for 20 min at 121°C. The cultures were maintained at 16 h photoperiod at 26°C temperature at a relative humidity of 70%. Uniformity of seeds were maintained by passing the seeds through standardized sieve. Each treatment was replicated four times, the LD50 dose was determined from number of seedlings that emerged or survived up to 56 days after sowing, and percent germination was calculated.

### Growth measurements

Seedlings height, internodal length, and length of primary roots was measured using a measuring scale while number of leaves was determined on visual observation basis. To study the comparative qualitative foliar symptoms, the severity of the plants condition was classified as follows: Plants without symptoms (WS), very mild chlorosis (VMC), partial mild chlorosis (PMC), mild chlorosis to burns on the margins and apices of the lower leaves (BMA), intense damage with leaf loss followed by plant death (SDD) and no germination (NG) that is, completely dead.

Tolerance index (TI) as given by La Rosa et al. (1989) was used to summarize the general effect of different concentrations of NaCl on rough lemon on the basis of reaction to salt treatment. The dry weight (DW) of the seeds/seedlings cultured on various concentrations of NaCl was measured after drying the samples at 70°C. The control treatment is designated as S10 for this parameter.

$$TI = 100 + \Sigma^n[X\ (T_x/T_o)\ 100]$$

Where, n is the Number of treatments; X is the salt concentration (1-9 g/l of NaCl), $T_x$, is the shoot/root weight of NaCl treated seedlings (1-9 g/l of NaCl) and $T_o$, is the shoot/root weight of untreated cuttings (g). The concentration of ions was expressed on percent dry weight basis.

### Chemical analysis

For chemical analysis, all the samples were washed with distilled water, dried at 70°C for 48 h to obtain constant weight and then ground. In the treatments beyond S5 (0.5%) where no germination took place, the swollen seeds were used for carrying out the analysis. The $Na^+$ and $K^+$ contents were determined by flame photometrically (Mapa, 1971) and $Cl^-$ by titration with silver nitrate approach (Gilliam, 1971).

### Statistical analysis

The data was analyzed with the Statistical Analysis System (SAS) V9.2 (SAS Institute, Cary, NC). LSD was used to compare the

**Table 1.** Rough lemon seed germination and growth parameters of *in vitro* grown rough lemon (*Citrus jambhiri* Lush) under saline conditions after 8 weeks.

| Treatment | *GRM | SMT** | SH (cm) | NOL | INL (cm) | LPR (cm) | FW† | DW† | SDW (g) | RDW (g) | S/R |
|---|---|---|---|---|---|---|---|---|---|---|---|
| S1-NaCl 17 mM | 89.3 | WS-VMC | 6.5 | 4.2 | 3.1 | 5.1 | 4.32 | 1.02 | 0.64 | 0.34 | 1.88 |
| S2-NaCl 35 mM | 88.7 | VMC-PMC | 6.1 | 3.3 | 3.1 | 5.9 | 3.89 | 0.92 | 0.60 | 0.31 | 1.94 |
| S3-NaCl 51 mM | 84.7 | PMC-BMA | 5.6 | 3.1 | 2.9 | 6.2 | 3.52 | 0.81 | 0.54 | 0.26 | 2.08 |
| S4-NaCl 68 mM | 69.7 | BMA | 3.8 | 3.0 | 1.1 | 6.6 | 2.12 | 0.62 | 0.42 | 0.19 | 2.21 |
| S5-NaCl 86 mM | 54.3 | BMA-SDD | 3.6 | 2.8 | 1.0 | 7.0 | 1.77 | 0.48 | 0.33 | 0.13 | 2.53 |
| S6-NaCl 103 mM | 0.00 | NG | 0.0 | 0.0 | 0.0 | 0.0 | 0.41 | 0.09 | - | - | - |
| S7-NaCl 120 mM | 0.00 | NG | 0.0 | 0.0 | 0.0 | 0.0 | 0.51 | 0.11 | - | - | - |
| S8-NaCl 137 mM | 0.00 | NG | 0.0 | 0.0 | 0.0 | 0.0 | 0.54 | 0.12 | - | - | - |
| S9-NaCl 154 mM | 0.00 | NG | 0.0 | 0.0 | 0.0 | 0.0 | 0.62 | 0.14 | | | |
| S10-NaCl 0mMControl | 100.0 | WS | 6.8 | 4.4 | 3.3 | 4.9 | 5.01 | 1.33 | 0.86 | 0.49 | 1.75 |
| LSD (P= 0.05%) | 2.88 | | 0.20 | 0.14 | 0.84 | 0.41 | 0.1 | 0.17 | 0.11 | 0.12 | 0.10 |

GRM, Germination percentage; SMT, symptoms; SH, seedling height; NOL, number of leaves per seedling; INL, internodal length; LPR, length of primary root; FW, fresh weight; DW, dry weight; SDW, stem dry weight; RDW, root dry weight; S/R, shoot to root ratio; **WS, plants without symptoms; VMC, very mild chlorosis; PMC, partial mild chlorosis; BMA, mild chlorosis to burns on margins and apices of the lower leaves; SDD, intense damage with leaf loss followed by plant death; NG, no germination; *, after 56 days; †, fresh and dry weight of seedlings (S1-S5 and Control) and seeds (S6-S9).

treatments with each other. The P value at 0.05 was used to find the significance between the treatments. Microsoft excel 2010 was used to make the figures. The correlation coefficient ($r^2$) value was used to evaluate the relationship between the salt treatment and growth parameters.

## RESULTS

### Treatment effect

Growth and nutrient acquisition in rough lemon were studied under *in vitro* conditions where salinity was induced by incorporating different concentrations (S1 to S9, Table 1) of salt (sodium chloride) in the culture media along with a control where no salt was added (Tables 1 and 2). There was significant effect on the survival and growth dynamics of rough lemon seedlings with gradual increase in concentration (NaCl). No seedling formation took place (Table 1) beyond S5 of salt concentration. The severity of symptom of NaCl injury can be evaluated from the data given in Table 1.

Maximum germination was found in control. Beyond S5, the seeds got swollen, sprouted but no seedling formation took place. The growth of the single shoot (measured as shoot length), number of leaves, and the internodal length of *C. jambhiri* under *in vitro* conditions was found to be maximum in control (Table 1). With increase in salt concentration in MS medium, the growth of single shoot under *in vitro* conditions decreased proportionally (Table 1). In comparison to the plant height, leaf number, and internodal length, the length of primary roots increased in proportion to the increase in salt concentration in the media and found positively correlated to increase in salt concentration until S5 after which no germination took place. The data depicted that total fresh weight as well as the dry weight of seedlings was found to be maximum in control. It decreased with increase in salt concentration treatment.

The dry weights of shoot and root were found significantly more in the control than other treatments whereas, minimum shoot and root dry weight was observed in S5 of NaCl (Table 1). Maximum shoot to root ratio was recorded in the S5 of NaCl treatment and minimum was observed in the control treatment. Nutrient uptake by the rough lemon seeds and seedlings is as shown in Table 2. Data in Table 2 clear the fact that in comparison to control, salt treatments resulted in increased levels of $Na^+$ and $Cl^-$ ions in seedlings including seeds, which got swollen but did not germinate. Maximum $Na^+$ and $Cl^-$ ions were observed in seeds with highest level of salt treatment. Compared to the control, $Na^+$ content in the seeds was almost 7 times while $Cl^-$ content was 9 times in the S9 treatment indicating that the concentration of $Cl^-$ was higher than that of sodium ($Na^+$) (Akilan et al., 1997).

It is evident from the Figure 1 that degree of tolerance index did not change with treatment and time, that is, the trend during 4 and 8 weeks of study was almost similar. Tolerance index was found minimum in control (S10). After 8 weeks of seed culturing in different salt treatments, changes in tolerance Index was more apparent where it increased to 391.04 in S5 of NaCl and then decreased to 308.95 in S6 of NaCl, while increasing to S10 in 154mM of NaCl treatment. It is interesting to observe that after 8 weeks, tolerance index from S1 to S5 treatments was higher while it was slightly lower for S5 to S10 of NaCl treatments than that observed after 4 weeks. Nevertheless, the trend was similar in both periods.

Figures 2a and 2b depict that the degree of injury increased with increase in concentration of salt in the

**Table 2.** Nutrition uptake of *in vitro* raised rough lemon (*Citrus jambhiri* Lush) seedlings/seeds under saline conditions (after 8 weeks).

| Treatment | Na (ppm) | K (ppm) | Cl (ppm) | K/Na | Na/Cl |
|---|---|---|---|---|---|
| S1-NaCl 17 mM | 18.2 | 32.8 | 40.4 | 1.4 | 0.45 |
| S2-NaCl 35 mM | 21.3 | 33.6 | 53 | 1.23 | 0.4 |
| S3-NaCl 51 mM | 21.8 | 34.2 | 55.8 | 1.23 | 0.39 |
| S4-NaCl 68 mM | 22.3 | 35.1 | 60.4 | 1.22 | 0.37 |
| S5-NaCl 86 mM | 22.9 | 35.7 | 61.3 | 1.21 | 0.37 |
| S6-NaCl 103 mM | 23.1 | 34.6 | 62.5 | 1.17 | 0.37 |
| S7-NaCl 120 mM | 23.4 | 33.5 | 63.6 | 1.11 | 0.37 |
| S8-NaCl 137 mM | 23.5 | 34.2 | 64.3 | 1.13 | 0.36 |
| S9-NaCl 154 mM | 23.8 | 32.4 | 65.6 | 1.06 | 0.36 |
| S10-NaCl 0 mMControl | 3.2 | 6.6 | 7.2 | 1.62 | 0.44 |
| LSD- (P= 0.05%) | 0.164 | 0.169 | 1.84 | NS | 0.36 |

*, Fresh and dry weight of seedlings (S1-S5 & Control) and seeds (S6-S9).

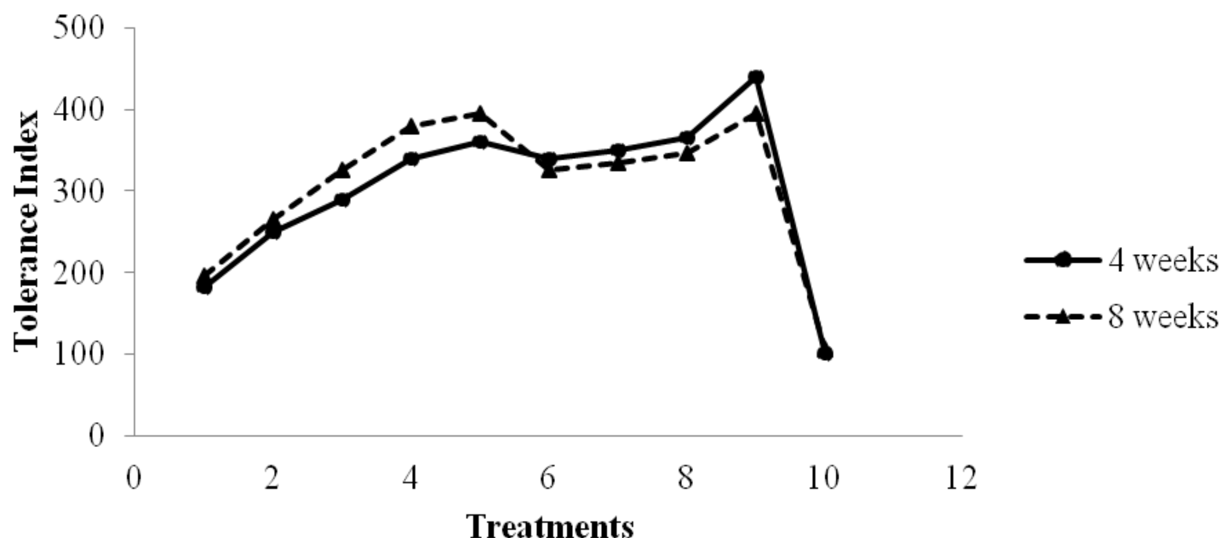


**Figure 1.** Tolerance index of rough lemon seeds and seedlings under salt conditions after 4 weeks and 8 weeks *in vitro* conditions.

culture media after 4 and 8 weeks, respectively. There was no injury (0 degree of injury) in control seedlings. Maximum degree of injury was observed in S9 of NaCl, might be due to high salt toxicity, where no germination took place and high concentration of sodium, potassium, and chloride found in the seeds. After 4 and 8 weeks, 3 degree level of injury was found in the S9 of NaCl treatment.

## Correlation and regression analysis

Correlation and regression coefficient were used to define the strength of relationship between the salt treatment and growth parameters as well as within growth parameters. The correlation between $Na^+$ and $Cl^-$ with $K^+$ was positive and significant (Table 3). It is evident from the Table 3 that there was strong correlation between the different growth parameters. It is being clear that with increase in $Na^+$ inside the plant tissue there is similar increase in $K^+$. The correlation between the germination, single shoot (measured as shoot length), number of leaves, and the internodal length, with salt treatment was negative and significant (Table 3). The correlation between fresh and dry weight with salt treatment was negative. High r2 value explained the strong relationship between the Na and K (Figure 3). In addition, Cl and K were found to have strong relationship with each other (Figure 4). However, negative correlation was found between the

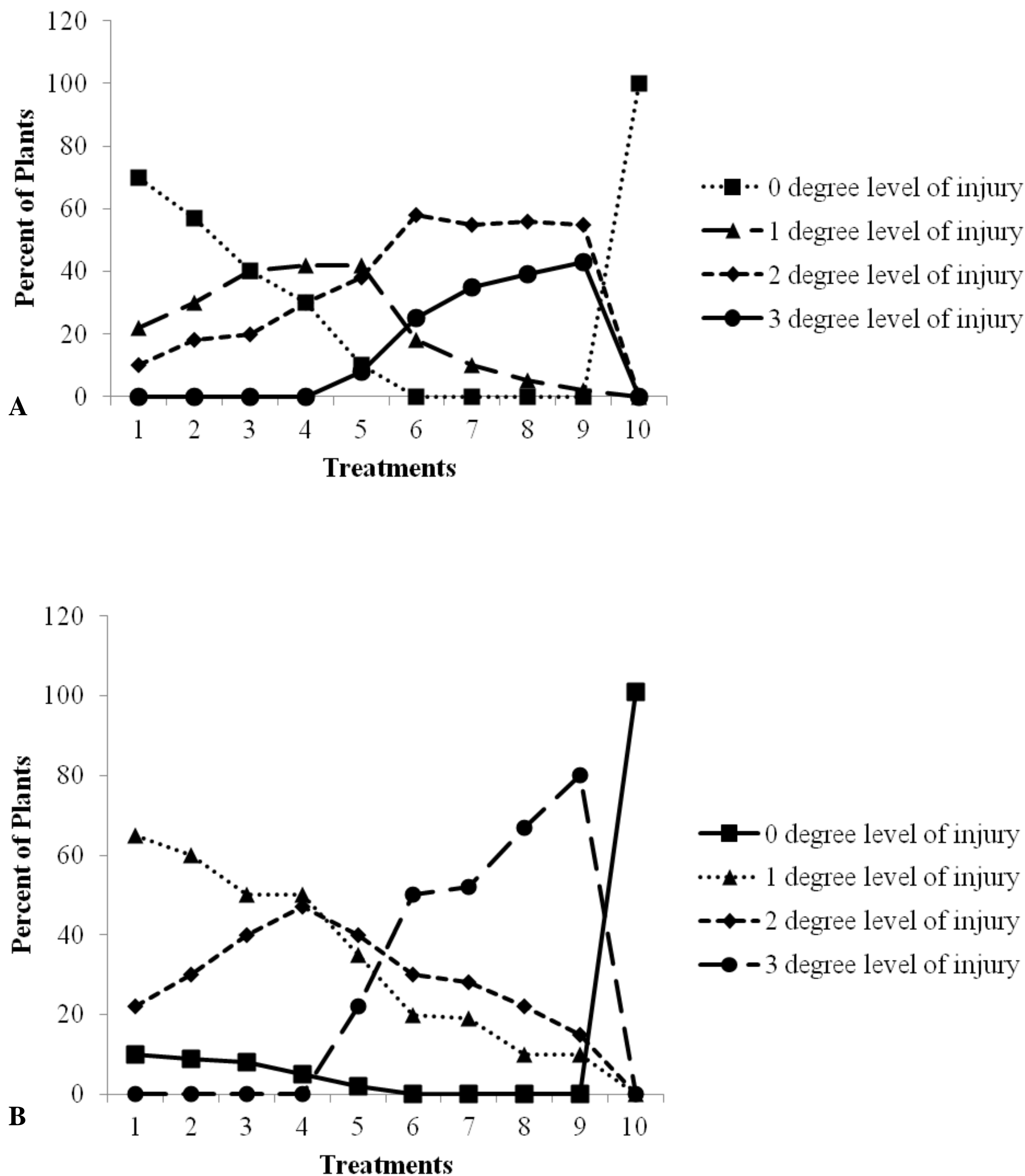


**Figure 2.** Degree of injuries in rough lemon seeds and seedlings after A, 4 weeks; B, 8 weeks.

salt treatments with fresh and dry weight of seeds and seedlings (Figure 5).

## DISCUSSION

The present investigation demonstrates the effects of increasing salt concentration on *C. jambhiri* seeds and seedlings. In all salt treatments (17 to 154 mM) leaves of rough lemon seedlings showed severe injury symptoms of chlorosis as well as necrosis which progressed fairly rapidly in the tips and along the leaf margins and then advanced basipetaly (Al-Yassin, 2004). The leaf injury symptoms were observed mostly in mature leaves. The seeds cultured in control treatment did not show any injury while the extent of chlorosis, necrosis and death shown by plants were in proportion to amount of NaCl added in the culture media (Sykes, 1985; Bongi and Loreto, 1989; Munns, 1993). Beyond 86 mM NaCl, the seeds got swollen, sprouted but no seedling formation took place (Yokas et al., 2008; Murkute, 2004). Increasing reverse osmotic pressure and concomitant salt toxicity could be the predisposal factor to reduce the physico-chemical processes in seed germination (Maathuis and Amtmann, 1999). It is evident from the results that NaCl treatments caused inhibition in plant growth due to

**Table 3.** Correlation between growth parameters and salt treatments.

| COR | Trt | FW | DW | Na (ppm) | K (ppm) | Cl (ppm) | K/Na | Na/Cl | SDW | RDW | S/R | GRM | SH | LPS | INL | LPR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Trt* | 1.00 | | | | | | | | | | | | | | | |
| FW* | -0.96 | 1.00 | | | | | | | | | | | | | | |
| DW* | -0.96 | 0.99 | 1.00 | | | | | | | | | | | | | |
| Na* | 0.70 | -0.71 | -0.76 | 1.00 | | | | | | | | | | | | |
| K* | 0.51 | -0.55 | -0.60 | 0.97 | 1.00 | | | | | | | | | | | |
| Cl* | 0.79 | -0.80 | -0.84 | 0.99 | 0.92 | 1.00 | | | | | | | | | | |
| K/Na* | -0.87 | 0.85 | 0.88 | -0.94 | -0.82 | -0.97 | 1.00 | | | | | | | | | |
| Na/Cl* | -0.88 | 0.89 | 0.87 | -0.75 | -0.59 | -0.85 | 0.88 | 1.00 | | | | | | | | |
| SDW* | -0.96 | 0.98 | 1.00 | -0.72 | -0.56 | -0.80 | 0.86 | 0.84 | 1.00 | | | | | | | |
| RDW* | -0.96 | 0.99 | 1.00 | -0.79 | -0.64 | -0.86 | 0.90 | 0.88 | 0.99 | 1.00 | | | | | | |
| S/R* | -0.75 | 0.73 | 0.76 | -0.30 | -0.12 | -0.38 | 0.52 | 0.47 | 0.81 | 0.73 | 1.00 | | | | | |
| GRM* | -0.94 | 0.96 | 0.96 | -0.58 | -0.40 | -0.67 | 0.76 | 0.76 | 0.98 | 0.95 | 0.89 | 1.00 | | | | |
| SH* | -0.95 | 0.98 | 0.97 | -0.60 | -0.41 | -0.70 | 0.78 | 0.81 | 0.98 | 0.96 | 0.86 | 0.99 | 1.00 | | | |
| LPS* | -0.94 | 0.94 | 0.96 | -0.61 | -0.42 | -0.70 | 0.80 | 0.79 | 0.97 | 0.94 | 0.90 | 0.99 | 0.98 | 1.00 | | |
| INL* | -0.94 | 0.99 | 0.96 | -0.62 | -0.45 | -0.72 | 0.77 | 0.85 | 0.96 | 0.96 | 0.74 | 0.95 | 0.97 | 0.92 | 1.00 | |
| LPR* | -0.75 | 0.73 | 0.75 | -0.28 | -0.10 | -0.36 | 0.50 | 0.45 | 0.81 | 0.73 | 1.00 | 0.89 | 0.86 | 0.89 | 0.74 | 1.00 |

COR, Correlation; Trt, treatment; FW, fresh weight; DW, dry weight, SDW, stem dry weight; RDW, root dry weight; S/R, shoot to root ratio; GRM, germination percentage; SH, stem height; LPS, length of primary shoot; INL, internodal length; LPR, length of primary root. *Correlation values were significant at 0.05, 0.01, and 0.001 level of significance.

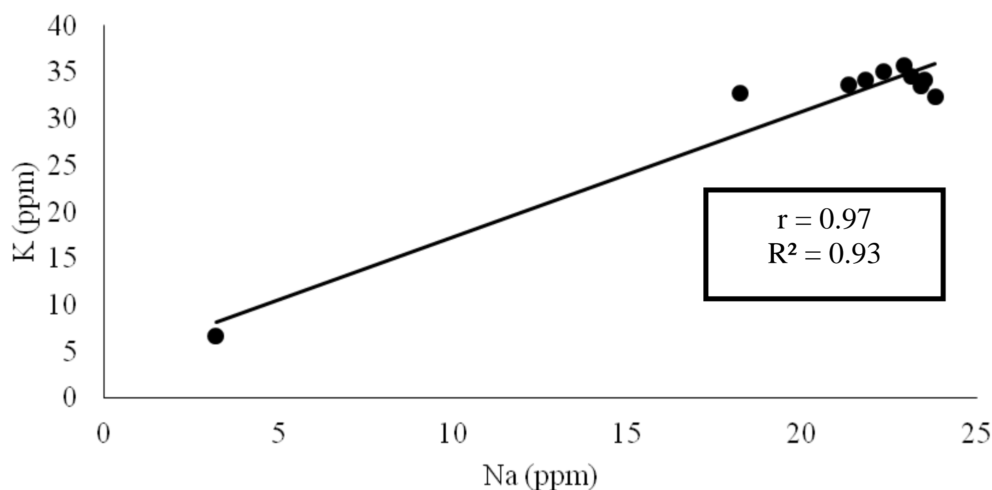


**Figure 3.** Regression and correlation between potassium and sodium level in rough lemon seed and seedlings.

decrease in proliferation ratio, fresh weight, shoot length, and number of leaves (Zidan et al., 1990; Pérez-Tornero et al., 2009). Since plant growth is a result of massive and irreversible expansion of young cells produced by ongoing merismetic divisions, salinization can inhibit both cell division and cell expansion in growing tissue of roots, stem and leaves thereby affecting shoot growth (Aazami et al., 2010; Forner-Giner et al., 2011). Under stress conditions, the *in vitro* grown seedlings tended to increase the root length for its survival (Abed et al., 2005) as compare to other fact. The effect of *in vitro* NaCl treatments on rough lemon supported the previous findings of *in vitro* NaCl treatments on grapevine cultivars (Downton and Millhouse, 1985).

Decrease in the number of leaves were not only due to the growth inhibiting effects of salt, but also due to the injurious effects of salt toxicity resulting in defoliation of the damaged leaves (Moya et al., 1999; Khoushbakht et al., 2010). First visual symptoms on the *in vitro* grown seedlings of rough lemon were withered shoot tips and

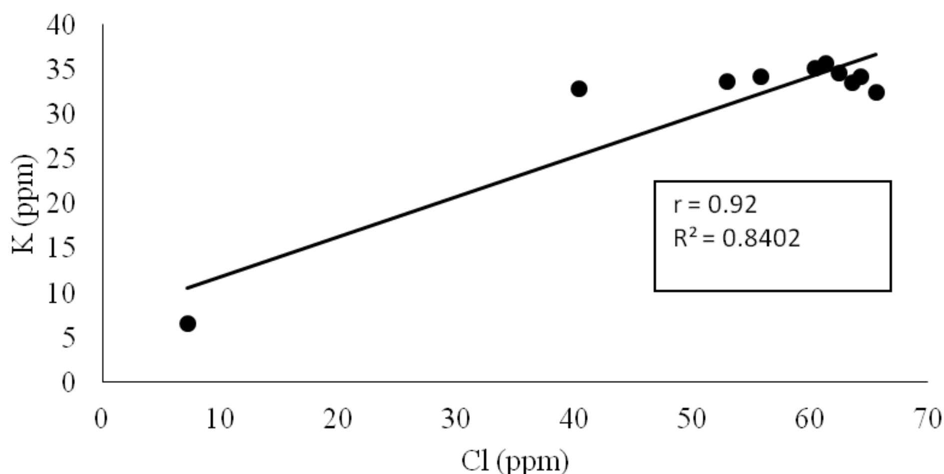


**Figure 4.** Regression and correlation between potassium and chloride levels in rough lemon seed and seedlings.

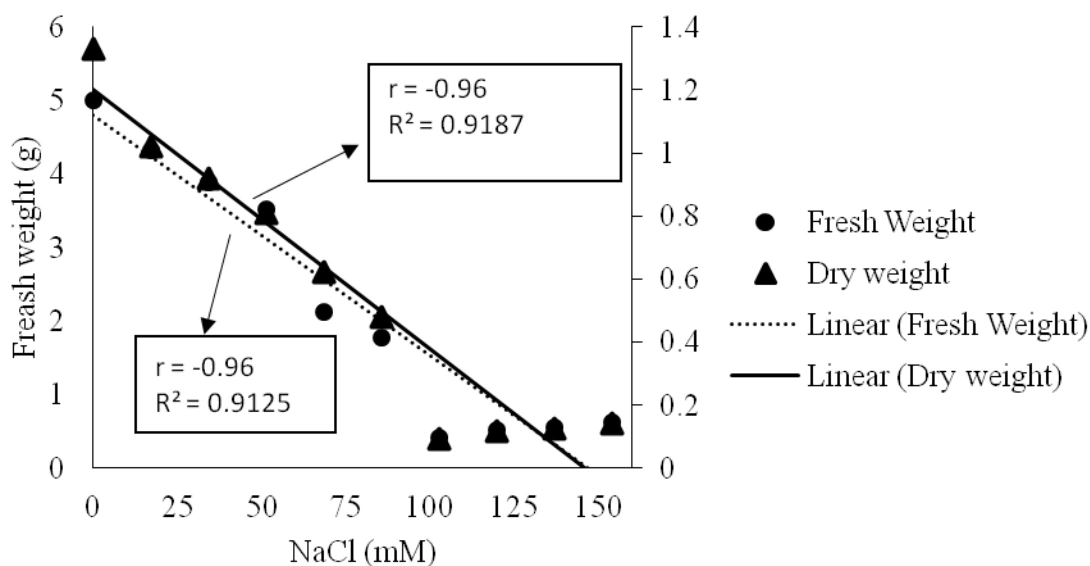


**Figure 5.** Regression and correlation between fresh and dry weights of rough lemon seed and seedlings with salt treatment.

leaf edges, which progressively increased inwards with increased in salinity levels and the length of treatment period (Yokas et al., 2008). These injurious effects progressed exponentially with increasing salinity levels.

In the present investigation, the reduced plant weight due to higher NaCl concentration and treatment period (8 weeks) can be attributed to the osmotic effect of salts in the soil that hinders plant growth and reduces production (Alkilan et al., 1997). As the citrus plant is not capable of excluding sodium from its system, sodium concentration builds up in the leaves thus causing leaf injury. The seeds cultured in treatments S6 to S9 (17 to 154 mM) did not germinate due to high salt concentration but got swollen and were used for mineral analysis. The growth inhibition due to salinity has been explained by suppression of nutrient ions absorption due to higher uptake of $Na^+$ and $Cl^-$ ions in competition with the nutrient ions (Alkilan et al., 1997). A portion of $Na^+$ was probably transported from the leaves through the phloem to root, relative to Cl, which accumulated in the leaves producing burns, nutriational imbalance, and reduced transpiration (Hassan and Catlin, 1984)

Potassium ion ($K^+$) is one of the most important solute which plays crucial role in maintaining the osmotic poten-

tial especially in roots. The $K^+/Na^+$ ratio decreased with the increase in NaCl concentration (Storey, 1995). Data demonstrates that salt treatments had a comparatively greater influence on the shoots compared to the roots. This was clearly evident in shoot to root ratio in the control seedlings (1.75), the ratio was less as compared to salt treated cultured media (LaRosa et al., 1989) Tolerance index for both 8 and 4 weeks salt treatment shows similar trend with slight variation. High tolerance index was shown by 8 week treatment for lower salt concentration from 17-86 mM. However, reverse pattern have been observed for high salt concentration (103 to 154 mM) than that observed after 4 weeks.

The study indicated that the addition of sodium chloride in the culture media decreased the osmotic potential of the media thus inducing salinity stress that adversely affected the *in vitro* germination capacity of seeds of *C. jambhiri*. The seeds of *C. jambhiri* did not germinate in the culture media containing salt beyond 103 mM of salt. Thus, the $LD_{50}$ value for seed germination of *C. jambhiri* was 0.5% NaCl in MS media.

## Conclusion

An inverse relationship was observed between the salt concentration and seed germination, seedling height, leaves, internodal length, root length. Salt injury was mostly on the mature leaves, which moved from tip to margin and then towards basipetal. The amount of chlorosis, necrosis, and death shown by seeds and seedlings were in proportion to amount of NaCl added into the culture media. In addition, the injurious effects of salt were exponential with increasing salinity levels. Salt had more adverse effect on the shoots than roots. Tolerance index with both 4 and 8 weeks salt treatment showed similar trend. Total fresh weight as well as the dry weight of seedlings decreased with an increase in salt concentration. Salt uptake was also increased with an increase in salt in the growing media. Selected seedlings were further evaluated under greenhouse conditions.

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# Genetic diversity in *Cucurbita pepo* landraces from northern KwaZulu-Natal, South Africa, revealed by random amplified polymorphic DNA (RAPD) markers

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**Genetic variation in seven selfed and unselfed *Cucurbita pepo* landraces from districts in KwaZulu-Natal Province was investigated using the random amplified polymorphic DNA (RAPD). Out of 36 primers screened nine were selected, which gave 100 clear and bright fragments, out of which 94 (94%) fragments were considered polymorphic. The sizes of bands ranged from 75 to 1800 bp. The number of bands per primer ranged between nine and 14. The genetic differentiation coefficient between populations ($G_{ST}$) varied between 0.0022 and 0.0100, while the gene flow ranged between 49.4545 and 223.7226. The effective number of alleles, Nei's gene diversity index and Shannon's information index were the highest in the selfed population from Zululand (yellow) district (ZS) population (Ne= 1.2046; H=0.1677; I=0.3060) and the lowest in unselfed population from uThungulu (yellow) (TNS) population (Ne=1.1512; H=0.1301; I=0.2518). The production of specific RAPD markers by different primers indicated gene diversity between: Selfed and unselfed populations from the same geographic origin; populations with yellow/orange mature fruit from a population with green mature fruit; and also among different populations in general. The selfed population from uThungulu (yellow) (TS) and TNS populations, both from uThungulu district, were the highest in genetic identity ($I_N$ = 0.9996) and the closest in the genetic distance (D = 0.0004). The unselfed population from Umkhanyakude (green) (CPSP) and unselfed population from Umkhanyakude (yellow (MNS) populations as well as CPSP and TNS populations were the lowest in genetic identity ($I_N$ = 0.9985) and the furthest in genetic distance (D = 0.0015). The dendrogram mainly grouped the populations according to their mature fruit colour, and then according to their geographical origin. All genetic parameters indicated that there was plentiful genetic diversity in *C. pepo* landraces of northern KwaZulu-Natal, South Africa.**

**Key words:** *Cucurbita pepo* landraces, genetic variation, self-pollination, random amplified polymorphic DNA (RAPD) markers, northern KwaZulu-Natal.

## INTRODUCTION

*Cucurbita pepo* is one of the most nutritionally and economically important species in the genus *Cucurbita* L. of Cucurbitaceae family that is cultivated worldwide and is of American origin (Tsivelikas et al., 2009; Ghobary and Ibrahim, 2010). Leaves of other wild Cucurbitaceae species indigenous to southern Africa, such as *Coccinia palmata* and *Lagenaria sphaerica* are cooked as leafy vegetables (Ntuli and Zobolo, 2008). *C. pepo* is a highly

polymorphic vegetable species, both in vegetative and reproductive characteristics, with a wide range of genetic variation occurring within it (Kathiravan et al., 2006; Formisano et al., 2012). Hadia et al. (2008) reported 84 and 87% polymorphism in *Cucurbita maxima* and *Cucurbita moschata*, respectively.

In traditional agriculture, genetic diversity is created by a diverse array of local varieties called landraces, which are well-adapted to local environmental conditions and inputs (Modi, 2004; Mujaju et al., 2010). Maintenance of landraces through *in situ* conservation is a preferred option for traditional small scale farmers (Modi, 2004). For example, part of the genetic variability of the first American summer squash cultigens remains intact in diverse landraces that are still cultivated for self-consumption and sale in local markets (Formisano et al., 2012).

In South Africa and other countries, communities and small scale farmers grow pumpkins as intercrop stands, whether with other plants, or with wild as well as cultivated forms of other cucurbits (Mujaju et al., 2010; Molebatsi et al., 2010; Torquebiau et al., 2010). This intercropping practice enhances the geneflow among the cucurbit species due to the random bee pollination (Cuevas-Marrero and Wessel-Beaver, 2008; Mujaju et al., 2010). Gene exchange among plant populations located in distant geographical areas can be influenced by occasional introduction of seeds and seedlings as well as informal seed exchanges among farmers (Yuan et al., 2007; Du et al., 2011; Barboza et al., 2012).

Self-pollination increases plant mean homozygosity, which is not the natural genetic state of cross-pollinated species and reduces the proportion of heterozygosity in the population thus reducing the vigor of plants (Ercan and Kurum, 2003; Cardoso, 2004). Traits studies by Ghobary and Ibrahim (2010) in selfed *C. pepo* showed that the phenotypic expression of these traits were indicative of their genetic behaviour. Among the different types of molecular markers available, random amplified polymorphic DNA (RAPD) are useful for the assessment of genetic diversity because of their simplicity, and are fast and easy to perform and comparatively cheaper than other markers and require no prior knowledge of DNA sequences (Dey et al., 2006; Hadia et al., 2008; Khan et al., 2009). The potential applications of RAPD fingerprinting in molecular biology include: Determination of taxonomic identities, detection of interspecific gene flow, assessment of kinship relationships, analysis of mixed genome samples and production of specific probes and and gene mutation (Hadrys et al., 1992; De Wolf et al., 2004). RAPD markers have been used extensively to analyze genetic diversity in cucurbits (Ferriol et al., 2003; 2004a; 2004b; Dey et al., 2006; Morimoto et al., 2006; Hadia et al., 2008; Khan et al., 2009; Tsivelikas et al., 2009; Du et al., 2011).

Some RAPD primers produce bands that are uniquely amplified in single accessions (Barracosa et al., 2008), thus ensuring diversity among investigated accessions. In the dendrogram, accessions from different localities can either group themselves according to their agro-climatic regions of origin or not. With amplified fragment length polymorphism (AFLP), the *C. maxima* accessions from America and Spain were clearly grouped according to geographic origin (Ferriol et al., 2004a), whereas the Spanish *C. moschata* accessions from different geographical origins clustered together with both sequence-related amplified polymorphism (SRAP) and AFLP markers (Ferriol et al., 2004b).

Although *Cucurbita* species are valued as traditional leafy vegetables in South Africa, no molecular work has analyzed genetic diversity on different cultivated landraces of this country. However, according to Yuan et al. (2007) genetic diversity of plant germplasm is the important basis of conservation biology and genetic improvement. The aim of the present work was to analyze the polymorphism and genetic diversity among and within unselfed and selfed *C. pepo* landraces from Umkhanyakude, uThungulu and Zululand districts of northern KwaZulu-Natal, South Africa using the RAPD markers.

## MATERIALS AND METHODS

### Plant material

The seeds of *C. pepo* landraces collected from uThungulu (Nkandla: 28°37'S 31°25'E), Umkhanyakude (Mseleni: 27°38'S 32°47'E) and Zululand (Ulundi: 28°32'S 31°47'E) districts were grown at the Experiment Station in the Botany Department, University of Zululand (Empangeni: 28°51'S 31°50'E), and were used as the source of plant material. Seven accessions (Table 1) constituted two sets of plants that were used to harvest leaf material for DNA extraction: One set (four accessions) was from the seeds that were directly from the communities of three districts, and another set (three accessions) was from the seeds that were initially from the communities of these districts but the fruits were self-pollinated to fix the traits in an accession, where the natural pollinators (bees) were suspected to mix some pollen as the communities are practicing intercropping with other Cucurbitaceae

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**Abbreviations: CPSP,** Unselfed population from Umkhanyakude (green); **MNS,** unselfed population from Umkhanyakude (yellow); **MS,** selfed population from Umkhanyakude (yellow); **TNS,** unselfed population from uThungulu (yellow); **TS,** selfed population from uThungulu (yellow); **ZNS,** unselfed population from Zululand (yellow); **ZS,** selfed population from Zululand (yellow) district; **RAPD,** random amplified polymorphic DNA; **AFLP,** amplified fragment length polymorphism; **SRAP,** sequence-related amplified polymorphism; **PCR,** polymerase chain reactions; **Ne,** effective number of alleles; **H,** Nei's gene diversity index; **I,** Shannon's information index; **ISSR,** inter-simple sequence repeat.

**Table 1.** Description of *Cucurbita pepo* landraces identified in three districts of northern KwaZulu-Natal.

| Code | Landrace name | Location (district) | Unselfed/ selfed | Mature fruit colour |
|---|---|---|---|---|
| MNS | iThanga | Umkhanyakude | Unselfed | Yellow/orange |
| MS | iThanga | Umkhanyakude | Selfed | Yellow/orange |
| TNS | iThanga | uThungulu | Unselfed | Yellow/orange |
| TS | iThanga | uThungulu | Selfed | Yellow/orange |
| ZNS | iThanga; iPhuzi | Zululand | Unselfed | Yellow/orange |
| ZS | iThanga; iPhuzi | Zululand | Selfed | Yellow/orange |
| CPSP | iNhlwathi emnyama | Umkhanyakude | Unselfed | Green |

species in their fields. Young (folded to semi-folded) leaves were picked, freeze-dried and stored at 4°C for future use.

**Self pollination procedure**

Three *C. pepo* accessions from Umkhanyakude, Uthungulu and Zululand districts were grown in different areas that were about 2 km away from each other to prevent the incidence of pollen transference among plants from different districts. Both pistillate and staminate flowers that were to be selfed the following morning were covered with a light fine-porous cloth (curtain fabric), mimicking a cheesecloth bag (Winsor et al., 2000), in the afternoon prior to flower anthesis. These were noticed by the appearance of a slight touch of yellow or orange at the apex of the corolla tube or rather when the yellow/orange colour of the petals (corolla) was clearly seen or intensified from the outside according to the procedure of Ercan and Kurum (2003). At flower anthesis, soon after dehiscence of pollen sacs (pollen anthesis), self pollination was initiated from 04h00 until about 08h30 in the morning, when the viability and germination potential of pollen grains was still high (Nepi and Pacini, 1993; Agbagwa et al., 2007) and both male and female flowers were wide open. During selfing, the staminate flowers were picked and had their corolla tubes removed to expose the pollen-laden stamens and the pollen was gentle rubbed on the stigma lobes of the pistillate flower in the same plant (Thralls and Treadwell, 2008; Fike, 2011). One male flower was used for each female recipient (Spencer and Snow, 2001) due to high levels of irregularities in anthesis of both staminate and pistillate flowers of one plant. To prevent uncontrolled bee pollination, after self-pollination, the pistillate flowers were re-covered for the whole day, and the cover was removed the following day, since the female flowers are receptive on the ovules for only one day of flower anthesis (Nepi and Pacini, 1993; Agbagwa et al., 2007).

**DNA extraction protocol**

The DNA from the freeze-dried, ground *C. pepo* leaves was extracted according to the manufacturer's instructions using commercially available DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA).

**Amount and purity of DNA**

The yield of DNA in ng/µl was measured using the Nano Drop ND-1000 spectrophotometer (software ND-1000 V3.5.1; USA). The DNA purity was calculated at 260/280 nm wavelengths, where the DNA with an absorbance ranging between 1.7 and 1.9 were considered pure and were used for the polymerase chain reactions (PCR).

**RAPD amplification**

Approximately 50 ng of DNA was amplified through the PCR using 25 µl reactions under the following conditions: 1X of GoTaq® Green Master Mix, 2X (Promega Corporation); 0.4 µM random 10-mer oligonucleotide primer (Inqaba Biotechnical Industries (Pty) Ltd), and Nuclease-Free Water (Promega Corporation).

Amplifications were performed in a MJ Mini Personal Thermal Cycler (from BIO-RAD, Sweden) programmed for an initial denaturation at 95°C for 5 min followed by 40 cylces of 95°C for 30 s, 36°C for 30 s, and 72°C for 1 min, and final extension of 72°C for 4 min. Amplified products were separated in 1% agarose in 1x Tris-Borate-EDTA (TBE) buffer with 125 ng ethidium bromide per liter, using gel electrophoresis run at 70V for 1 h. The nucleic acid markers 100 bp (Promega Corporation) and 1 kb (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd) were used to compare the amplification product sizes.

Of 36 primers tested, nine primers producing distinct polymorphic bands were selected for further analysis (Table 2). Each primer producing constituent amplification of well defined, brightly staining bands were used in further amplification of DNA from all individuals. Amplification was repeated to ensure reproducibility of scored products. RAPD markers were scored for presence or absence, and each marker was identified by primer and marker size.

The polymorphism rates of RAPD primers were evaluated using seven *C. pepo* landrace populations: Umkhanyakude unselfed (MNS); Umkhanyakude selfed (MS); Umkhanyakude green ripe fruits (CPSP); uThungulu unselfed (TNS); uThungulu selfed (TS); Zululand unselfed (ZNS); and Zululand selfed (ZS).

**Data analysis**

The data for RAPD was analysed using the Population Genetic Analysis (POPGENE version 1.31) (Yeh et al., 1999). The following genetic diversity parameters were determined: 1) The number of polymorphic bands (A) and the percentage of polymorphic bands (P); 2) the effective number of alleles per loci (Ne); 3) gene diversity (H) and Shannon's information index (I); 4) Nei's genetic distances (D) and genetic identity ($I_N$), which were evaluated using the cluster analysis that was performed with the unweighted pair group method of arithmetic average (UPGMA); and 5) the coefficient gene differentiation among the populations within species, which was determined using Nei's gene diversity method. The formula was

$$G_{ST} = D_{ST}/H_T,\ H_T = H_S + D_{ST}$$

Where, $H_T$ is the total gene diversity, $H_S$ is the gene diversity within the population, and $D_{ST}$ is the gene diversity between populations. The gene flow was determined as $Nm = 0.5\ (1\text{-}G_{ST})/G_{ST}$ (Yuan et al., 2007).

**Table 2.** Sequence, produced band size range and polymorphism of different RAPD primers, as well as genetic variability within seven *Cucurbita pepo* populations.

| RAPD Primer | Sequence (5' – 3') | Band size range (bp) | N | A | P | Ne | H | I | $H_S$ | $G_{ST}$ | Nm |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CB9 | GGTGACGCAG | 100-1300 | 10 | 9 | 90 | 1.1216 | 0.1084 | 0.2201 | 0.1081 | 0.0027 | 183.3149 |
| CB12 | AGTCGACGCC | 100-1300 | 9 | 8 | 89 | 1.1650 | 0.1417 | 0.2707 | 0.1412 | 0.0031 | 160.5899 |
| CB13 | ACGCATCGGA | 100-1100 | 10 | 10 | 100 | 1.1539 | 0.1334 | 0.2584 | 0.1331 | 0.0022 | 223.7226 |
| CB15 | GGCTGGTTCC | 75-1400 | 12 | 11 | 92 | 1.2169 | 0.1783 | 0.3227 | 0.1772 | 0.0060 | 82.5414 |
| CB17 | GTAACCAGCC | 100-1400 | 12 | 11 | 92 | 1.2167 | 0.1781 | 0.3225 | 0.1774 | 0.0039 | 127.1654 |
| CB19 | GGTGCTCCGT | 75-1400 | 14 | 14 | 100 | 1.1540 | 0.1335 | 0.2585 | 0.1330 | 0.0035 | 143.1361 |
| CB21 | CAGCACTGAC | 100-1800 | 12 | 12 | 100 | 1.1937 | 0.1623 | 0.3004 | 0.1611 | 0.0070 | 70.7242 |
| CB23 | CTGGGCACGA | 200-1400 | 11 | 9 | 82 | 1.2293 | 0.1865 | 0.3340 | 0.1847 | 0.0100 | 49.4545 |
| CB27 | AAGTGCGACC | 200-1300 | 10 | 10 | 100 | 1.1541 | 0.1335 | 0.2586 | 0.1329 | 0.0047 | 106.6980 |
| Total | - | - | 100 | 94 | 94 | | | | | | |
| Average | - | - | 11.11 | 10.44 | 94 | 1.1784 | 0.1506 | 0.2829 | 0.1499 | 0.0051 | 97.7840 |

CB, Cucurbita; N, total number of bands; A, number of polymorphic bands; P, percentage of polymorphism; Ne, effective number of alleles; H, Nei's gene diversity; I, Shannon's information index; $H_{S,}$ genetic diversity index within populations; $G_{ST,}$ genetic differentiation coefficient between populations; Nm, gene flow.

## RESULTS

### Polymorphism of RAPD amplified bands by different primers

The analysis of seven *C. pepo* populations with nine RAPD primers identified a total of 100 reproducible fragments (Table 2). Among them, 94 were polymorphic (94%), ranging in size from 75 to 1800 bp. Between nine and 14 fragments were amplified per primer, with an average of 11.11 fragments. Maximum number (14) of polymorphic fragments was obtained with the primer CB19. The number of polymorphic fragments for each primer varied from eight and 14, with an average of 10.44 fragments.

### Population genetic diversity, differentiation ($G_{ST}$), and gene flow (Nm)

The effective number of alleles (Ne) estimated the reciprocal of homozygosity as ranging from 1.1216 (CB9) to 1.2293 (CB23), with an average of 1.1784 ± 0.0370. The Nei's gene diversity index (H) varied from 0.1084 (CB9) to 0.1865 (CB23), with an average of 0.1506 ± 0.0267. The Shannon's information index (I) ranged from 0.2201 (CB9) to 0.3340 (CB23), with an average of 0.2829 ± 0.0386. The genetic diversity index within populations ($H_S$) varied from 0.1081 (CB9) to 0.1847 (CB23), with an average of 0.1499 ± 0.0007.

The genetic differentiation coefficient between populations ($G_{ST}$) ranged between 0.0022 (CB13) and 0.0100 (CB23), with an average of 0.0051, which showed that the genetic variation between populations accounted between 0.22 and 1.00%, with an average of 0.51% of the total variation. The gene flow (Nm) ranged between 49.4545 (CB23) and 223.7226 (CB13), with an average of 97.7840, according to the genetic differentiation coefficient between populations, which indicated that there was a high exchange between *C. pepo* populations.

The effective number of alleles (Ne), Nei's gene diversity index (H) and Shannon's information index (I) were conducted to further understand the genetic diversity among the selfed and unselfed populations of *C. pepo* originating from three different districts (Table 2). The effective number of alleles, Nei's gene diversity index and Shannon's information index were the highest in ZS population (Ne= 1.2046; H=0.1677; I=0.3060) and the lowest in TNS population (Ne=1.1512; H=0.1301; I = 0.2518) (Table 3). Comparisons between selfed and unselfed populations within a district revealed that selfed populations of uThungulu and Zululand districts had higher effective number of alleles, Nei's gene diversity index and Shannon's information index than their analogous unselfed populations, while the opposite was evident in populations from Umkhanyakude district.

### Specific RAPD marker production per primer per landrace(s)

Specific RAPD markers for CPSP population only were produced by: primers CB9 and CB12 (700 bp); primers CB13, CB19 and CB21 (1000 bp); and primer CB27 (1100 bp). Also primers CB15 and CB17 produced exclusive markers 100 and 500 bp, respectively, in all populations except CPSP population. The CPSP population had fruits that did not change their colour to orange or yellow at maturity.

Primer CB9 also showed the effect of selfing by

**Table 3.** Genetic variation among *Cucurbita pepo* populations**.**

| Population | Effective number of alleles (Ne) | Nei's gene diversity (H) | Shannon's information index (I) |
|---|---|---|---|
| MNS | 1.2009 ± 0.0773 | 0.1643 ± 0.0523 | 0.3003 ± 0.0733 |
| MS | 1.1651 ± 0.0661 | 0.1393 ± 0.0469 | 0.2645 ± 0.0681 |
| TNS | 1.1512 ± 0.0482 | 0.1301 ± 0.0349 | 0.2518 ± 0.0512 |
| TS | 1.1724 ± 0.0409 | 0.1461 ± 0.0302 | 0.2760 ± 0.0449 |
| ZNS | 1.1688 ± 0.0566 | 0.1427 ± 0.0391 | 0.2704 ± 0.0556 |
| ZS | 1.2046 ± 0.0655 | 0.1677 ± 0.0438 | 0.3060 ± 0.0613 |
| CPSP | 1.1901 ± 0.0440 | 0.1587 ± 0.0318 | 0.2941 ± 0.0467 |

Values are Mean ± standard deviation. MNS, Umkhanyakude unselfed; MS, Umkhanyakude selfed; TNS, uThungulu unselfed; NS, uThungulu selfed; ZNS, Zululand unselfed; ZS, Zululand selfed; CPSP, *C. pepo* species with dark green mature fruits.

**Table 4.** Nei's original measure of genetic identity and genetic distance among seven *C. pepo* populations.

| Population ID | MNS | MS | TNS | TS | ZNS | ZS | CPSP |
|---|---|---|---|---|---|---|---|
| MNS | **** | 0.9995 | 0.9988 | 0.9992 | 0.9986 | 0.9989 | 0.9985 |
| MS | 0.0005 | **** | 0.9988 | 0.9991 | 0.9987 | 0.9991 | 0.9992 |
| TNS | 0.0012 | 0.0012 | **** | 0.9996 | 0.9994 | 0.9990 | 0.9985 |
| TS | 0.0008 | 0.0009 | 0.0004 | **** | 0.9994 | 0.9990 | 0.9993 |
| ZNS | 0.0014 | 0.0013 | 0.0006 | 0.0006 | **** | 0.9990 | 0.9988 |
| ZS | 0.0011 | 0.0009 | 0.0010 | 0.0010 | 0.0010 | **** | 0.9989 |
| CPSP | 0.0015 | 0.0008 | 0.0015 | 0.0007 | 0.0012 | 0.0011 | **** |

Nei's genetic identity (above diagonal) and genetic distance (below diagonal). MNS, Umkhanyakude unselfed; MS, Umkhanyakude selfed; TNS, uThungulu unselfed; NS, uThungulu selfed; ZNS, Zululand unselfed; ZS, Zululand selfed; CPSP, *C. pepo* species with dark green mature fruits.

identifying a unique band of 400 bp in unselfed populations from all districts including CPSP population, while primer CB21 identified marker 900 bp for only unselfed populations from all districts excluding CPSP population. Also CB15 produced specific band (200 bp) for only selfed populations from all districts, but also including the CPSP population. Primer CB9 produced unique band 600 bp in MS and ZS populations only, which were both selfed but from different districts. Primer CB23 produced a specific band 1000 bp in MNS and MS populations, both from one eco-geographic region, Umkhanyakude district.

The following specific RAPD markers amplified bands only in each of the following populations: MNS population [CB9 (800 bp), CB12 (900 bp), CB27 (800 bp)]; TNS population [CB23 (900 bp), CB27 (1000 bp)]; MS population [CB9 (1200 bp)] and TS population [CB13 (900 bp)].

## Genetic identity and genetic distance between *Cucurbita pepo* populations

To further elucidate the gene differentiation among *C. pepo* populations, Nei's original measure of genetic identity ($I_N$) and genetic distance (D) was calculated (Table 4). The genetic identity ranged from 0.9985 to 0.9996, while the genetic distance varied from 0.0004 to 0.0015. The TS and TNS populations were the highest in genetic identity ($I_N$ = 0.9996) and the closest in the genetic distance (D = 0.0004). The CPSP and MNS populations as well as CPSP and TNS populations were the lowest in genetic identity ($I_N$ = 0.9985) and the furthest in genetic distance (D = 0.0015).

The phylogenetic relationship between populations was further illustrated by a dendrogram (Figure 1) using the UPGMA algorithm based on Nei's genetic distance (1972). The dendrogram grouped the populations into two main clusters, where cluster two had CPSP population (landrace with dark green and light green variegation at maturity) which was distant from a group with all other six populations. As with the results obtained in cluster one, which had two sub-clusters, a clear grouping according to geographical origin was observed. Sub-cluster one grouped the populations from Umkhanyakude district (MNS and MS). Sub-cluster two included populations from Uthungulu (TNS and TS) and Zululand (ZNS and ZS) districts, where populations from the former district formed a cluster and then assembled with ZNS and ZS

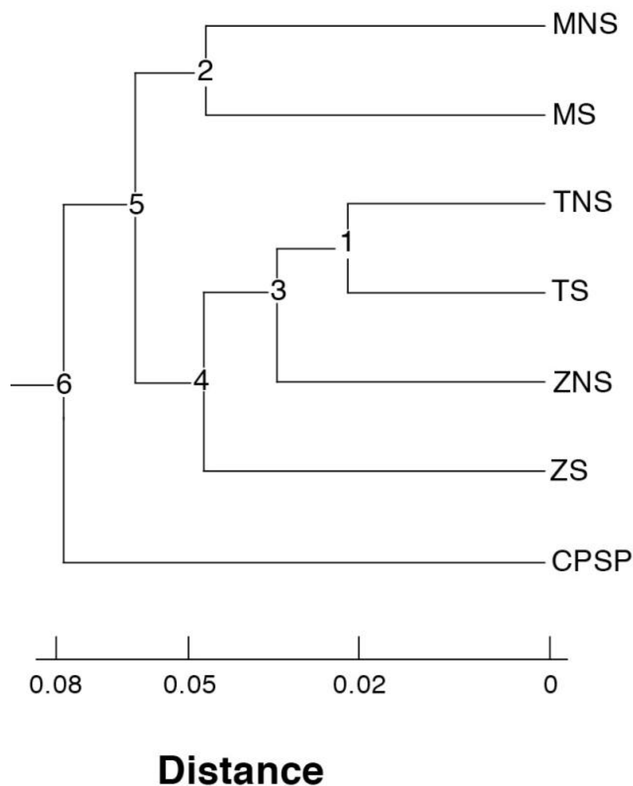


**Figure 1.** Unweighted pair group method of arithmetic mean (UPGMA) dendrogram based on Nei's (1972) genetic distance, summarizing data on differentiation in seven populations of *C. pepo* eco-geographical populations. MNS, Umkhanyakude unselfed; MS, Umkhanyakude selfed; TNS, uThungulu unselfed; NS, uThungulu selfed; ZNS, Zululand unselfed; ZS, Zululand selfed; CPSP, *C. pepo* species with dark green mature fruits.

populations, both from Zululand district.

## DISCUSSION

### Polymorphism in *C. pepo* populations

The level of polymorphism among the *C. pepo* population was relatively high, ranging between 82 and 100% with an average of 94% (Table 2). This high level of RAPD markers polymorphism in *C. pepo* genotypes is in accordance with the results of Kathiravan et al. (2006) Hadia et al. (2008) and Tsivelikas et al. (2009), who reported that *C. pepo* is a highly polymorphic species. In an earlier study, Hadia et al. (2008) reported 89% polymorphism among *C. pepo* genotypes.

### Population genetic structure and geographic diversity of northern KwaZulu-Natal *C. pepo* landraces

The average value of $G_{ST}$ was 0.0051, based on the RAPD markers for *C. pepo* in northern KwaZulu-Natal (Table 2), indicating that the gene differentiation was higher within population (99.49%) than between the populations (0.51%). Gene differentiation and gene flow are important indices to evaluate the population genetic structure. The gene differentiation coefficients of *C. pepo* landraces, mainly from Spain, were 0.25 and 0.18, when analysed with SRAP and AFLP, respectively (Ferriol et al., 2003). Further, the gene differentiation coefficients of Spanish *C. moschata* landraces as determined by AFLP and SRAP analyses were 0.28 and 0.17, respectively (Ferriol et al., 2004b).

Based on the RAPD analysis of *C. pepo* landraces of northern KwaZulu-Natal, the average value of the gene flow was 97.7840 (Table 2), which according to Han et al. (2007) was overwhelming the effect of genetic drift because its value was above four. According to Yuan et al. (2007) and Mujaju et al. (2010), the movement of genes within and between populations, the gene flow, is negatively correlated with the gene differentiation, and is transferred by pollen and seed between populations for seed plants.

Also, the population genetic structure is mainly affected by a long distance diffusion of pollen and diffusion capability of pollen offspring owing to inbreeding and outcrossing propagation (Yuan et al., 2007). However, *C. pepo* distributed in different districts of KwaZulu-Natal with long geographical distance, had a very low possibility of the pollen spread by insects (particularly bees) between populations. Several authors have reported the evidence of gene flow and hybridization between several interplanted *Cucurbita* species (Decker-Walters et al., 1990; Wessel-Beaver, 2000; Montes-Hernandez and Eguiarte, 2002; Cuevas-Marrero and Wessel-Beaver, 2008).

The possible gene flow among *Cucurbita* species was also possible for long distances' pollen transfer by bees ranging between 800 and 1300 m (Montes-Hernandez and Eguiarte, 2002; Spencer and Snow, 2001). Therefore the main way of gene exchanges can be occasional introduction of seeds as enhanced by seed exchanges between farmers of different districts in KwaZulu-Natal (Montes-Hernandez and Eguiarte, 2002; Ferriol et al., 2004a; 2004b; Yuan et al., 2007; Mujaju et al., 2010; Du et al., 2011; Barboza et al., 2012).

The effective number of alleles (Ne=1.1784), Nei's gene diversity index (H=0.1506), Shannon's information index (I=0.2829), and genetic diversity index within populations ($H_S$=0.1499), also indicate molecular genetic diversity for the *C. pepo* populations studied herein (Table 2). The gene diversity obtained with RAPD markers among different *C. pepo* landraces from northern KwaZulu-Natal was less than that obtained with AFLP markers among different *C. moschata* accessions (Wu et al., 2011); with RAPD among *Trichosanthes dioica* accessions (Khan et al., 2009); and also with inter-simple sequence repeat (ISSR), SRAP and RAPD markers among Turkish and Foreign *Cucumis melo* genotypes (Yildiz et al., 2011). The production of a specific RAPD marker by primer CB23 (1000 bp)

in both MNS and MS populations from Umkhanyakude district indicated the effect of eco-geographic differences in gene diversity of plants, where landraces from the same geographic area are closely related (Ferriol et al., 2004a,b; Du et al., 2011).

### Genetic variation between unselfed and selfed *C. pepo* populations

The higher effective number of alleles, which estimates the reciprocal of homozygosity, in selfed populations than unselfed populations from both uThungulu and Zululand districts (Table 3), showed the effect of self-pollination by enhancing homozygosity while reducing the heterozygosity in plant genomes, concurring with the reports by Ercan and Kurum (2003), Cardoso (2004) and Ferrari et al. (2006; 2007). However the higher Nei's gene diversity index and Shannon's information index in these selfed populations (Table 3) disagreed with these reports, but on the contrary the selfed population from Umkhanyakude district, showed this increase in homozygosity with selfing. This greater genetic variability among these selfed populations, particularly from uThungulu and Zululand districts, can increase their effectiveness of selection and the amount of genetic improvement in a breeding program as suggested by Ghobary and Ibrahim (2010).

The production of specific RAPD markers for either selfed or unselfed populations only, also confirmed that selfing had changed the genetic state of these *C. pepo* landraces from different districts of northern KwaZulu-Natal as reported earlier in other *Cucurbita* species (Ercan and Kurum, 2003; Cardoso, 2004; Ferrari et al., 2006; 2007).

### Specific RAPD marker per *C. pepo* landrace(s)

The production of unique bands only in MNS population [CB9 (800 bp), CB12 (900 bp), CB27 (800 bp)]; TNS population [CB23 (900 bp), CB27 (1000 bp)]; MS population [CB9 (1200 bp)] and TS population [CB13 (900 bp)], indicated the genotype variation among *C. pepo* landraces in northern KwaZulu-Natal. Hadia et al. (2008) identified the specific RAPD markers that showed genotypes variation among *C. maxima*, *C. moschata* and *C. pepo* species, as well as those showing differences within their populations. Again, Barracosa et al. (2008) in their study of *Ceratonia siliqua*, made use of unique RAPD markers that were cultivar-specific to differentiate the Portuguese cultivars.

Six primers (CB9; CB12; CB13; CB19; CB21 and CB27) produced unique bands for CPSP population only, while two primers (CB15 and CB17) produced unique bands for all other six populations except CPSP. The absence and presence of these unique bands in CPSP populations only indicated that these primers were probably marking the genes or loci that affect fruit colour development or change at maturity among these landraces, where the CPSP maintains its green fruit variegation at maturity. Paris (2000) reported 11 loci that have been identified as affecting developmental fruit colour in *C. pepo*, and of these, three genes of major effect – D, l-1, and l-2 – account for a considerable portion of the genetic variation in intensity of fruit colouration that is observed in this species. The developmental fruit colouration from light green fruits, several days past anthesis, except for some darkening of the main capillary veins, becoming blackish-green past anthesis, and then turn intense orange on ripening (Paris, 2000; 2009), as observed in other landraces, was conferred by genotype D/D l-1/l-1 L-2/L-2 (Paris, 2000). However, fruits of L-1/ — L-2/ — plants are intense green throughout development, as the case with the CPSP landrace, where, in contrast to plants homozygous recessive for either or both l genes, the fruits of L-1/— L-2/ — plants retain their black-green colour through maturity, not turning orange or yellow when ripe (Paris, 2000).

### Genetic identity and genetic distance of *Cucurbita pepo* between populations

The range of genetic identity from 0.9985 to 0.9996 and genetic distance range from 0.0004 to 0.0015 indicated the presence of variability among the seven populations of *C. pepo* in northern KwaZulu-Natal (Table 4). The TS and TNS populations were the highest in genetic identity ($I_N$ = 0.9996) and the closest in the genetic distance (D = 0.0004), probably because they originate from the same district, but CPSP and TNS populations were the lowest in genetic identity ($I_N$ = 0.9985) and the furthest in genetic distance (D = 0.0015), because they were collected from distant geographical regions, where most of the farmers have maintained the productions of these landraces for many years. The same was reported among *Punica granatum* cultivars (Yuan et al., 2007), *T. dioica* accessions (Khan et al., 2009) and *C. moschata* accessions (Du et al., 2011; Barboza et al., 2012). However CPSP and MNS populations, both from Umkhanyakude district, were also the lowest in genetic identity ($I_N$ = 0.9985) and the furthest in genetic distance (D = 0.0015), possibly because CPSP populations do not change fruit colour to orange or yellow at maturity whereas MNS population do change. Therefore the differences in genes that are responsible for colour formation at maturity as explained by Paris (2000; 2009) might have influenced this low genetic identity and wide genetic distance between these populations.

A clear grouping, first according to fruit colour change at maturity, and secondly according to geographical origin, was obtained (Figure 1). The separation of CPSP population from a group of six populations that changed in their fruit colour at maturity may support the wide genetic variation in these landraces with reference to their fruit colour formation as recorded by Paris (2000; 2009). A separate sub-cluster of Umkhanyakude district populations

(MNS and MS) from those of uThungulu (TNS and TS) and Zululand (ZNS and ZS) districts showed that these populations are grouped according to their agro-ecological regions. This concurs with the findings of Ferriol et al. (2004a,b;) in their study of *C. maxima* and *C. moschata*, respectively, who obtained the results with both SRAP and AFLP markers, where accessions were grouping according to their geographical origin. Also, Amadou et al. (2001) in their genetic diver-sity analysis of *Vigna subterranea* using RAPD markers, found the highest similarity on accessions that were originating from the same country. Further, Tsivelikas et al. (2009) report the genetic diversity analysis of *C. moschata* landraces using RAPD markers where acces-sions were grouped according to the agro-climatic regions and not according to the morphological traits.

Further, the grouping of population from Uthungulu district with those from Zululand district was probably due to existence of seed exchanges among farmers of these districts. The same was reported earlier by other researchers in cucurbits (Montes-Hernandez and Eguiarte, 2002; Ferriol et al., 2004a; 2004b; Barboza et al., 2012) and other species (Yuan et al., 2007).

## Conclusion

The RAPD analysis discovered sufficient variations among the *C. pepo* landraces. This marker revealed the genetic diversity in landraces with differences in fruit colour change at maturity at a higher extent, and then landraces with different geographical origin. It further confirmed the effect of selfing on the change of plant's genetic make-up.

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# An alternative safer and cost effective surface sterilization method for sugarcane (*Saccharum officinarum L.)* explants

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**Regardless of its serious health effect, mercury chloride is frequently utilized for surface sterilization to mitigate microbial contamination in sugarcane tissue culture. The current study aimed at finding an alternative safer and cost effective sterilization method to substitute mercury chloride. In the study, sugarcane shoot tip blocks were treated with three concentrations (1, 3, and 5% active ingredient of chlorine) of local bleach (Berekina) for varying exposure time (10, 15, 20, 25 and 30 min). Surface sterilization with 0.1% mercury chloride for 10 min was used as standard check. Combinations of the surface sterilization treatments were applied to explants of two sugarcane genotypes in completely randomized design. Data were collected on contamination and survival percentage of explants after 15 days of *in vitro* culturing on Murashige and Skoog (MS) medium supplemented with 2 mg l-1 6-benzylamino purine + 0.5 mg l-1 indole-3- butyric acid. Data were subjected to three way analysis of variance. The study verified that surface sterilization with Berekina 5% ingredients of chlorine for 25 min exposure time is optimal for sugarcane shoot tip decontamination and this treatment combination can replace sterilization with 0.1% mercury chloride for 10 min.**

**Key words:** Berekina, mercury chloride, exposure time, shoot tip, *in vitro*.

## INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a monocot-tyledonous crop plant that belongs to the family Poaceae (Sharma, 2005; Cha-um et al., 2006). It has chromosome number of 2n = 80 (Daniels and Roach, 1987; Asano et al., 2004). It is a tall perennial crop that tillers at the base, grows three to four meters tall and about five cm in diameter (Singh, 2003). Today, the crop is grown in over 110 countries and 50% of the production occurs in Brazil and India (FAO, 2008). The 2010/11 global annual sugar production was estimated to be more than 174.3 million tons (Czarinikow, 2010) and sugarcane accounts nearly for 70% of the production (Sengar, 2010). Sugarcane has been cultivated in the tropical and subtropical regions of the world for its multiple uses. The sugar juice is used for making sugar (Cox et al., 2000). Molasses (thick syrupy residue) is used in the production of ethanol (blended for motor fuel) and as livestock feed. The bagasse (fibrous portion) is burned to provide heat and electricity for sugar mills and green tops can be used as livestock feed (Mackintosh, 2000).

Sugarcane is one of the commercial cash crops of Ethiopia. Teklemariam (1991) stated that sugar industry development in Ethiopia has great contribution to the development of the livelihood of the society and the national economy in many ways. These contributions are concerned with production and consumption of sugar

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incomegeneration, employment creation, revenue contribution, foreign exchange earnings and savings, electric power contribution, skill and know how development, capital formation, agriculture and other industries development, urbanization and marketing development benefits. Hence, improving sugarcane production capacity has a paramount importance in enhancing the economic prosperity of the country.

Varieties of sugarcane are highly heterogeneous and generally multiplied vegetatively by stem cutting. However, the conventional seed cane production method where stem cuttings with two or three nodes are used as planting material, has various limitations. The seed multiplication rate is too low (1:6 to1:8) which makes the spread of newly released varieties slow, taking over 10 years to scale up a newly released variety to the commercial level (Cheema and Hussain, 2004; Sengar, 2010), and also facilitates the spread of pathogens and may result in epidemics (Schenck and Lehrer, 2000). Moreover, the method requires large nursery space: one hectare nursery for 10 to 15 hectares field planting (Sundara, 2000). Therefore, it is imperative to find out and implement a technological intervention that circumvents the problems associated with the conventional propagation methods.

Plant tissue culture (micropropagation) is a tool for obtaining rapid, mass multiplication of disease free, true to type planting material (Singh, 2003). Ali et al. (2004) stated that sugarcane micropropagation has the benefits of rapid propagation of new cane varieties, reduction in seed use, regeneration of large number of true to type plantlets from a small tissue, elimination of pathogens and storage of plant germplasm under aseptic condition. Explants surface sterilization is one of the critical steps in plant tissue culture.

During sterilization, the living materials should not lose their biological activity and only contaminants should be eliminated; explants need to be surface sterilized only by treatment with disinfectant solution at suitable concentrations for a specified period (Oyebanji et al., 2009). In line with this, mercury chloride solution is frequently utilized to mitigate microbial contamination in sugarcane tissue culture (Ali et al., 2004; Gosal et al., 2006; Behera and Sahoo, 2009; Kanwar, 2009; Lal et al., 2009).

However, mercuric chloride ($HgCl_2$) is reported to be a widespread environmental and industrial pollutant, which induces severe alterations in the tissues of both animals and men (Lund et al*.,* 1993; Mahboob et al., 2001). Various reports depicted that mercury chloride results in a variety of undesirable health effects including neurological, renal, respiratory, immune, dermatological, reproductive and developmental sequela (Risher and Amler, 2005; WHO, 2005; Sharma et al., 2007; Durak et al., 2010).

Akin-Idowu et al. (2009) also stated that mercury chloride is difficult to dispose off. Hence, it is imperative to find an alternative safer surface sterilant that can replace the highly deleterious environmental pollutant mercury chloride. In line with this, the use of locally available bleach or Berekina makes the sterilization process simple, rapid and cost effective (WHO, 2006; Oyebanji et al., 2009). Thus, targeting to standardize both the concentration and length of exposure time for the local bleach (Berekina) for surface sterilization of sugarcane explants helps to sterilize explants at reasonable cost and in safer condition. Therefore, the present study was initiated to develop an alternative safer and cost effective surface sterilization procedure for aseptic shoot tip culture of sugarcane.

## MATERIALS AND METHODS

The study was conducted at plant tissue culture laboratory of Jimma University College of Agriculture and Veterinary Medicine, Ethiopia. Two sugarcane genotypes, Co449 and Co678, were used in this study. They were obtained from Matahara Sugar Estate, operating under Ethiopian Sugar Corporation. To reduce contamination from explants, the stock plants were raised by planting seed canes under greenhouse condition. For *in vitro* studies, shoot tips were excised from tops of three to four months-old actively growing sugarcane raised in the greenhouse. The leaves were removed and the shoot blocks were taken to the laboratory. In the laboratory, surrounding leaf sheaths were carefully removed one by one until the inner white sheaths were exposed. Then, 10 cm long tops were collected by cutting off at the two ends, locating the growing point somewhere in the middle of the top. The shoot tip blocks were washed under running tap water for 30 min with soap solution and treated with 0.3% kocide (fungicide solution) for one and half hour under laminar air flow cabinet. After decanting kocide solution, shoot tip blocks were washed three times with sterile distilled water and further immersed in 70% ethanol for 30 s and rinsed three times with sterile distilled water to remove ethanol.

To increase efficacy, two drops of Tween-20 solution was added into Berekina and $HgCl_2$ solutions. Tween-20 solution is a wetting agent added to the disinfectants to reduce surface tension and allow better surface contact. Decanting the sterilizing solutions under safe condition, the explants were washed three times each for 5 min with sterile distilled water and left for 10 min to make the surface dry. Thereafter, leaf sheaths damaged during sterilization were removed using sterilized forceps. Finally, 2 cm long shoot tips were excised with sterilized scalpels and cultured on MS basal medium supplemented with 2 $mgl^{-1}$ benzyl amino purine (BAP) + 0.5 $mgl^{-1}$ indole-3-butyric acid (IBA), 3% sucrose, and 8% agar (Bakesha et al.,2002). Cultures were transferred to growth chamber with environmental conditions: Temperature of 25 ± 2°C, 16 h light photoperiod, relative humidity of 70-80%, and fluorescent light intensity of 2500 lux. For the two sugarcane genotypes (Co449 and Co678), three concentrations levels of Berekina (1, 3 and 5% active ingredient of chlorine) and five levels of exposure time (10, 15, 20, 25 and 30 min) with a treatment combination of 2 x 3 x 5 = 30 plus one standard check (0.1% (w/v) mercury chloride ($HgCl_2$) for 10 min). Experiments were set up in a completely randomized design (CRD). Fifteen explants were randomly assigned to each treatment combination. One explant was used per culture jar. Data were recorded on the number of contaminated and survived (clean) cultures per treatment combinations after 15 days of inoculation or culturing. The data were converted into percentages and subjected to three way analysis of variance using Statistical Analysis System (SAS) software version 9.2 (SAS Institute Inc., 2008). Statistical significance was computed at a 5% probability level and treatment means were separated using procedure of REGWQ (Ryan, Elinot, Gabriel, and Welsh)- multiple range test.

**Table 1.** ANOVA summary for the effect of Berekina and explant exposure time on contamination and survival level of cultured explants.

| Source of variation | DF | Mean square | |
|---|---|---|---|
| | | Contamination percentage | Survival Percentage |
| Gen | 1 | 23.85$^{ns}$ | 24.20$^{ns}$ |
| Ber | 2 | 19621.47*** | 14994.57*** |
| Time | 4 | 5224.96*** | 4074.57*** |
| Gen * Ber | 2 | 14.07$^{ns}$ | 13.83$^{ns}$ |
| Gen *Time | 4 | 19.41$^{ns}$ | 16.79$^{ns}$ |
| Ber * Time | 8 | 446.20*** | 346.42*** |
| Gen * Ber *Time | 8 | 5.19$^{ns}$ | 6.42$^{ns}$ |
| CV (%) | | 4.06 | 7.91 |

***, Very highly significant ($P \leq 0.0001$) at $\alpha=0.05$ significance level; ns, non significant ($p > 0.05$) at $\alpha=0.05$ significance level; DF, degree of freedom; Gen, sugarcane genotypes; Ber, Berekina (local bleach).

**Table 2.** Effect of different concentrations of Berekina and length of exposure time on *in vitro* sugarcane explants contamination and survival percentage.

| (v/v) % of Chlorine in Berekina | Time of Exposure (minutes) | Explant percent | |
|---|---|---|---|
| | | Contaminated | Clean and survived |
| 1 | 10 | 100.00$^{a}$ | 0.00$^{j}$ |
| 1 | 15 | 93.33$^{b}$ | 6.67$^{i}$ |
| 1 | 20 | 86.67$^{c}$ | 13.33$^{h}$ |
| 1 | 25 | 80.00$^{d}$ | 20.00$^{g}$ |
| 1 | 30 | 73.33$^{e}$ | 26.67$^{f}$ |
| 3 | 10 | 93.33$^{b}$ | 6.67$^{i}$ |
| 3 | 15 | 80.00$^{d}$ | 20.00$^{g}$ |
| 3 | 20 | 73.33$^{e}$ | 26.67$^{f}$ |
| 3 | 25 | 60.00$^{f}$ | 40.00$^{e}$ |
| 3 | 30 | 53.33$^{g}$ | 46.67$^{d}$ |
| 5 | 10 | 73.33$^{e}$ | 26.67$^{f}$ |
| 5 | 15 | 53.33$^{g}$ | 46.67$^{d}$ |
| 5 | 20 | 40.00$^{h}$ | 60.00$^{c}$ |
| 5 | 25 | 13.33$^{i}$ | 86.67$^{a}$ |
| 5 | 30 | 6.67$^{j}$ | 66.67$^{bc}$ |
| Control ($HgCl_2$) | 10 | 13.33$^{i}$ | 73.33$^{b}$ |
| CV ( %) | | 4.06 | 7.91 |

Means in column with the same letter are not significantly different by Ryan-Einot-Gabriel-Welsch Multiple Range Test at $\alpha$ = 5% significant level.

## RESULTS AND DISCUSSION

Analysis of variance (ANOVA) revealed that the main effect, concentration of Berekina (Ber) and time of explant exposure (Time), and the interaction effect of concentration of Berekina by length of time of exposure (Ber*Time) had very highly significant ($p < 0.0001$) effect on both the contamination and survival level of sugarcane shoot tip *in vitro* culture (Table 1). The ANOVA also showed that genotype had non-significant ($p>0.05$) effect on establishment of aseptic culture indicating that shoot tip surface sterilization using Berekina does not depend on sugarcane genotypes.

The highest explant contamination (100%) and the least culture survival (0%) were recorded when explants were surface sterilized with 1% active chlorinated Berekina for 10 min (Table 2). This might be due to the insufficiency of the concentration of active chlorine in Berekina and length of exposure time to kill culture contaminants. Fungi and bacteria are the most commonly

observed culture contaminants. The least culture contamination (13.33%) and the highest culture survival (86.67%) were recorded when explants were decontaminated with 5% active chlorinated Berekina for 25 min, which is similar rate of culture contamination (13.33%) but greater rate of culture survival compared with those treated with standard check, 0.1% mercury chloride ($HgCl_2$) for 10 min (Table 2). Lesser survival rate (73.33%) of culture treated with the standard check might be due to the phytotoxic effect of mercury chloride.

It was also observed that an increase in the percentage of active chlorine in Berekina from 1 to 5% when explants treated for 10 min decreased the culture contamination by 26.67% and increased the culture survival by the same percentage. Whereas an increase in exposure time from 10 to 30 min for 1% concentration of chlorine in Berekina had decreased culture contamination to 73.33% and increased culture survival by 26.67%. Generally, it was observed that as the concentration of chlorine in Berekina increased from 1 to 5% and the time of exposure increased from 10 to 25 min, the percent of contamination decreased and the percent of culture survival increased. This might be because of synergetic effect of chlorine concentration and length of exposure time that had a killing effect on culture contaminants.

However, surface sterilization with 5% active chlorinated Berekina beyond 25 min resulted in less contamination but more death of explants. This could be due to the phytotoxic effect of 5% chlorinated Berekina at longer exposure time. This implies that during sterilezation, the living materials(explants) should not lose their biological activity and only contaminants should be eliminated; explants are surface sterilized only by treatment with disinfectant solution at suitable concentrations for a specified period (Oyebanji et al., 2009). Therefore, in the present study local bleach or Berekina with 5% ingredients of chlorine for 25 min exposure time was found to be optimal for sugarcane shoot tip sterilization and this treatment combination can replace sterilization with 0.1% mercury chloride for 10 min.

The current result is consistent with the findings of Ali et al. (2004) who reported 60-75% contamination free cultures as best results when explants of two wild relatives of sugarcane (Erianthus 3854 and SES 089) are treated with 0.1% $HgCl_2$ for 10 min but disagree with the results of Yilekal (2011) who reported 72.23% contamination free culture using 5% active chlorinated Berekina for 20 min. The deviation might be due to the fact that the author did not expose explants to Berekina beyond 20 min and also due to difference in explant type.

Tesfaye (2011) reported 60% culture survival rate using 5% active ingredient of Berekina for 20 min which was exactly similar to the result obtained in the present study at the same (5%) concentration of Berekina for 20 min explant exposure time. Chaudhry et al. (2007) also reported 70-90% contamination control (culture growth) using clorox (commercial bleach) in surface sterilization of apical meristem of three sugarcane cultivars: HSF-240, CP-77-400 and CPF-230. Therefore, Berekina (with 5% active ingredient of chlorine) which is affordable, widely available in local shops and supermarkets, environmentally friendly, less toxic compared to $HgCl_2$, and does not require special handling and waste disposal precautions (Emongor et al 2010) can be used to surface sterilize sugarcane explants particularly shoot tips. Surface sterilization with local bleach is a safer option for both researchers and the environment (Emongor et al 2010).

## Conclusion

Based on the current result, it is possible to deduce that, we have developed a safer and cost effective alternative procedure for surface sterilization of sugarcane explants, which can use Berekina as surface sterilizing agent as a substitute for mercury chloride. Hence, Berekina with 5% active ingredient of chlorine for 25 min exposure time is the appropriate combination to use for surface sterilization of sugarcane shoot tips.

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# Influence of plant growth regulators on indirect shoot organogenesis and secondary metabolite production in *Aconitum violaceum* Jacq.

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**Influence of plant growth regulators on indirect regeneration and secondary metabolite production in *Aconitum violaceum* Jacq. was evaluated. Among the different plant growth regulators studied, 2.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.25 µM kinetin (Kn) promoted the highest frequency of callus production for indirect regeneration. 6-Benzyl aninopurine (BAP) was more effective in improving shoot regeneration and secondary metabolite production compared to thidiazuron (TDZ). The highest frequency of regeneration (61.8%) was obtained when calli were transferred to Murashige and Skoog medium supplemented with 1 µM BAP and 0.5 µM α-naphthalene acetic acid (NAA) and was more than two-times higher when compared to the treatments with cytokinin only. Supplementation with low NAA concentrations resulted reduction in *in vitro* secondary metabolite production in most cases, when compared to treatments with cytokinin only. Moreover, differences in cytokinin concentrations significantly affected secondary metabolite production in some cases. The current findings highlighted the differential effects of auxin-cytokinin interactions on indirect shoot regeneration and the production of secondary metabolites in *A. violaceum*.**

**Key words:** Cytokinins, auxins, plant tissue culture, indirect regeneration, *aconitum violaceum,* secondary metabolites.

## INTRODUCTION

*Aconitum violaceum* Jacq. member of family Ranunculaceae is an important medicinal plant found in subalpine and alpine areas of Indian Himalayan Region at 3500-4000 m elevations, and shares its position with threatened plant species of IHR (Chaudhary and Rao, 1998; CAMP, 2003). Roots of this plant are the natural source of alkaloid aconitine, a neurotoxin which attributes to the medicinal properties of the plant (Anonymous, 1988). The crude extract of underground parts possess antipyretic and analgesic properties and traditionally been used in renal pain, rheumatism, high fever and for the treatment of snake and scorpion bites, contagious infections and inflammation of the intestines (Kirtikar and Basu, 1984; Ameri, 1998; Chauhan, 1999).

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**Abbreviations: MS medium,** Murashige and Skoog medium; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **TDZ,** thidiazuron; **BAP,** 6-benzylamino purine; **NAA,** α-naphthalene acetic acid; **Kn,** kinetin; **IAA,** indole-3- acetic acid; **PGRs,** plant growth regulators; **HPLC,** high performance liquid chromatography.

Due to increased demand from pharmaceutical industries, uncontrolled collection and lack of organized cultivation, plant is going depleted in natural habitat. Therefore, to protect the natural germplasm and to collaborate with rising demand of the plant material there is an immense need of biotechnology based interventions to ascertain secure conservation of elite germplasm. *In vitro* propagation techniques have contributed significantly to the growth of pharmaceutical industry over the past several decades in a variety of ways including varietal improvement. The use of *in vitro* techniques for rapid and mass propagation offers possibilities for 'recovery' of endangered species, thus reducing the risk of extinction (Nadeem et al., 2001).

Regeneration from secondary meristems is useful for both these approaches because it facilitates the production of nonchimeric plants. The indirect regeneration of adventitious shoots is an alternative method to somatic embryogenesis in obtaining whole plant regeneration of explants. Shoot organogenesis and somatic embryogenesis from various explants have been reported earlier (De Wit et al., 1990; Kintzios et al., 1999; Vergne et al., 2010). One of the major factors affecting the success of *in vitro* plant propagation is the choice of plant growth regulators (PGRs). As a result, PGRs (especially cytokinins and auxins) are often added to culture media for the purpose of controlling different physiological responses *in vitro*, leading to the production of tissues (such as callus), organs (such as shoots and roots) or whole plants.

In spite of extensive hype in worldwide research interests in medicinally important plants and pharmaceutically active compounds there from, no conventional scientific attempts have so far been reported to conserve *A. violaceum*. The reports on *in vitro* multiplication and conservation of genetic diversity of genus *Aconitum* provides a realistic insight of its medicinal importance (Cervelli, 1987; Shiping et al., 1988; Giri et al., 1993; Watad et al., 1995; Giri et al., 1997; Mitka et al., 2007; Hatwal et al 2011). Thus, due to uncontrolled exploitation, the risk of loss of natural genetic resource of *A. violaceum* and biodiversity conservation aspects has drawn the attention in current research. Keeping above points in mind, the present study was aimed to investigate the role of plant growth regulators in improving/optimizing indirect shoot regeneration in *A. violaceum*. The influence of an auxin-cytokinin interaction on secondary metabolite production *in vitro* was also studied.

## MATERIALS AND METHODS

### Plant material and culture conditions

Plants of *A. violaceum* Jacq. were collected from Hemkund (30° 41'55" N to 79° 47' E 4100 m amsl, District Chamoli, Uttarakhand) of Garhwal Himalaya during the month of September. Plants were brought to the laboratory and used immediately for *in vitro* propagation following the protocol of Mishra-Rawat et al. (2013). Shoot tips and nodal segments of the plants were used as explants for the establishment of *in vitro* cultures. Surface sterilization of the explants was done with 0.5% Bavistin (30 min) and 0.1% mercuric chloride solution (2 min) followed by four times subsequent washing with sterile water. The sterile explants were cultured on MS (Murashige and Skoog, 1962) basal medium containing 0.8% (w/v) agar and sucrose (3% w/v) without any plant growth promoters (Figure 3). The pH of the medium was adjusted to 5.8 before autoclaving. Cultures were maintained at 25 ± 2°C in 16/8 h light/dark cycle on racks fitted with cool fluorescent tubes (Philips 40 W; 42.0 and 60.0 μmol/m$^2$/s irradiance inside and outside the culture flasks, respectively). Sub-culturing was carried out at three to four weeks interval for optimal growth.

### Callus induction, maintenance and plant regeneration

Three types of explants (leaf, shoot tip and nodal segment, taken from the same mother plant) were inoculated on the MS medium supplemented with 0.5 to 10.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.25 to 2.0 μM kinetin (Kn), 3% (w/v) sucrose and 0.8% agar for callus induction. Callus cultures were maintained through subculturing after every 30 days. Consequently, calli subcultured within three generations were used to induce adventitious shoots. Adventitious shoot induction medium was MS medium supplemented with various concentration of 6-benzyl aninopurine (BAP), thidiazuron (TDZ) and α-naphthalene acetic acid (NAA) (0.5 to 5.0 μM BAP, 0.5 to 5.0 μM TDZ and 0.25 to 2.0 μM NAA: single or in combination) and ultimately plants were regenerated. Regenerated shoots were cultured up to eight weeks and further used for secondary metabolite analysis. Culture conditions were same as described above for shoot induction.

### Experimental design and statistical analysis

Each experiment was repeated three times and each repeat had seven replicates. Data were analyzed in a factorial based on completely randomized design (CRD). Data were analyzed using statistical programs MSTAT-C and SPSS. Statistically significant averages were compared using Duncan's Multiple Range tests. Graphs were plotted with the Excel program. Differences were regarded as significant at P ≤0.05.

### Active ingredient analysis

Ten gram (FW) of plant material of each treatment was used for analysis. Plants were washed to remove media particle and dried at room temperature (25°C) for 20 days. The air dried material were powdered and made into a composite mixture before chemical analysis. Extraction of active ingredients was done following the method of Hikino et al. (1983). The powdered samples (1.0 g) were extracted (25 ml x 3; 30 min each) with ammoniacal ether (ether containing 5% v/v, ammonia solution); the residue was then extracted with methanol (25 ml) for 16 h followed by two more extractions for 3 h each.

### Column chromatography

Samples were further purified on neutral alumina (Sisco Research Laboratories Pvt Ltd., Mumbai) columns (8 x 2 cm; length and diameter) eluted with 50 ml of ethyle acetate and methanol (7.3, v/v). The eluates were dried *in vacuo* (30°C) in a rotary film evaporator, dissolved in high performance liquid chromatography (HPLC) grade methanol (1.0 ml) for further analysis by HPLC.

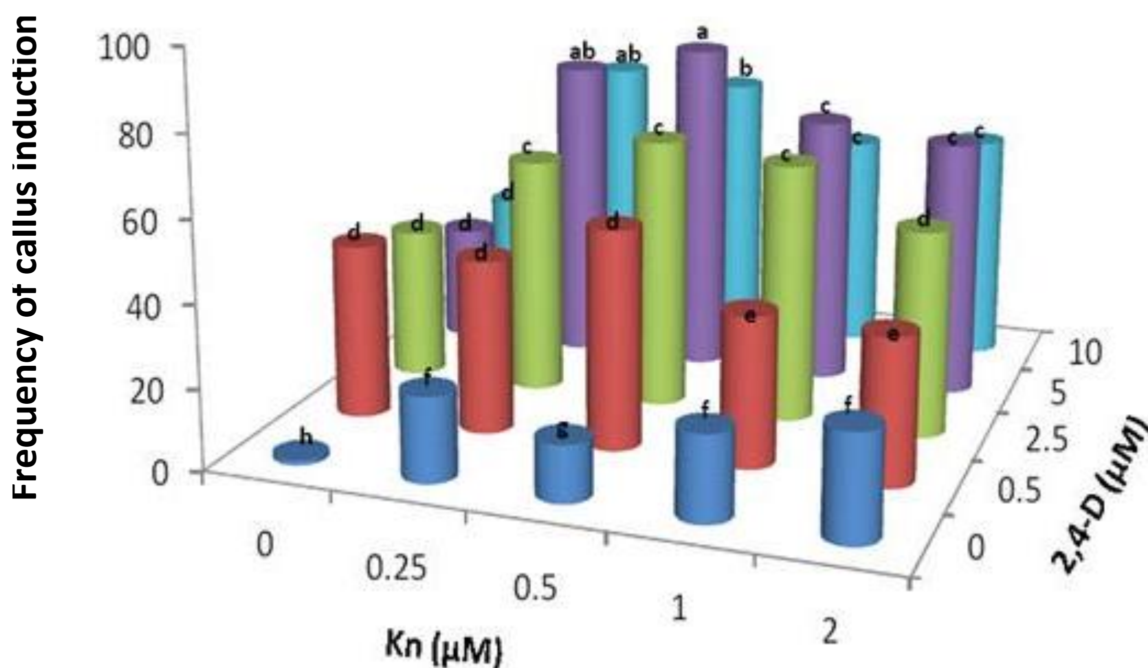


**Figure 1.** Effects of different concentration of 2,4-D and Kn on Indirect regeneration (somatic embryogenesis and organogenesis) of *Aconitum violaceum*. Means with different letters are significantly different according to Duncan's Multiple Range Test (P≤0.05).

#### High performance liquid chromatography (HPLC)

The quantification of aconitine was carried out using a HPLC system (Shimadzu corporation, Japan; Model LC-10 ATVP) in RP-1 Spherisorb column (250 x 4.6 mm id, 5 µm; Merck Darmstadt, Germany), eluted in an isocratic mode with methanol and water (60:40, v/v) containing 0.1% of acetic acid. The column elutes were monitored using an online UV detector set at 263 nm. The peaks were identified on the basis of retention time and quantification was carried out on peak area basis using a dose- response curve prepared with authentic compounds. Three analyses were done per sample. The lower limit of detection was approximately 100 ng. Aconitine was obtained from Sigma chemicals Co. St. Louis, USA.

## RESULTS

### Callus induction and maintenance

Callus induction started with enlargement of the exposed surface and cut regions of the explants (leaves, shoot tips and nodal segments), 21-28 days after the initial culture. All types of explants produced callus on all callus induction media. A greater proportion of explants on medium with 5.0 µM 2,4-D and 0.5 µM Kn initiated callus (leaves, 83.1%, shoot tips, 24.1%, nodal segments, 39.0%) than other media (data not shown). As the leaf explants showed maximum callus formation, further experiment regarding the effect of types and concentration of plant growth regulators on indirect regeneration was carried out with callus induced from leaf explants only (Figure 1). Media containing 5.0 µM 2,4-D and 0.5 µM Kn produced embryogenic globular and light green callus (Figure 2a), whereas media containing 10.0 µM 2,4-D produced soft watery callus creamy or light brown in color (Figure 2b).

### Effect of cytokinins on regeneration of adventitious shoots from callus

The calli generated from different callus induction media were transferred to regeneration medium. The highest frequency of regeneration (61.8%) was obtained from media containing 1 µM BAP and 0.5 µM NAA (Figures 2c and 3a). The media containing TDZ showed lesser number of shoots compared to the media containing BAP (Figure 3a). Figure 3b shows the effects of different types and concentrations of cytokinins alone or in combination with NAA on shoot production after 8 weeks of culture. Increased shoot production was observed in the medium supplemented with cytokinin and auxin when compared to the PGR-free treatment in all the cases (Figure 3b). Maximum shoot production was observed with medium supplemented with 1 µM BAP and 0.5 µM NAA (10.3 ± 0.8) which was more than three times higher than the control (2.9 ± 0.3), although this was not significantly different from the result of the treatment with 2.5 µM TDZ and 0.5 µM NAA (8.1 ± 0.3). The highest shoot fresh weight was recorded in the treatment with 2.5 µM BAP

**Figure 2.** Indirect regeneration in *A. violaceum.* A, Formation of embryogenic callus on MS medium supplemented with 5.0 µM 2,4-D and 0.5 µM Kn; B, white brown callus formation on MS medium supplemented with 10.0 µM 2,4-D only; C, organogenesis in MS medium supplemented with 1 µM BAP and 0.5 µM NAA.

and 0.25 µM NAA (Figure 3c).

## Active ingredient analysis

The active ingredient content of regenerated shoots from different cytokinin treatments singly or in combination with NAA concentrations are presented in Figure 3d. The aconitine content recorded in treatments containing cytokinin alone or in combination was significantly higher compared to that of the PGR-free medium in most of the cases. An increase in NAA concentration gave increased aconitine content in some cases. The supplementation of medium with cytokinins and low NAA concentrations (0.25 µM) resulted in significantly reduced aconitine content in most cases (Figure 3d). Maximum secondary metabolite production (0.87% of dry weight) was recorded in the medium supplemented with BAP and NAA (2.5 µM BAP +1.0 µM NAA). These results supposedly suggest a possible antagonistic interaction of low NAA concentrations with cytokinin on aconitine production *in vitro*, in this plant species.

## DISCUSSION

The indirect regeneration of adventitious shoots is an alternative method to somatic embryogenesis in obtaining whole plant regeneration of explants. In the present study, all the three types of explants produced callus with 5.0 µM 2,4-D and 0.5 µM Kn. Initiated callus were embryogenic and light green in colour. The auxin 2,4-dichlorophenoxyacetic acid (2,4-D) has been employed (single or in combination) in induction of somatic embryogenesis in various plant species (Rout et al., 1991; Dohm et al., 2001; Li et al., 2002; Estabrooks et al., 2007). Increasing 2,4-D concentration from 0.5 to 10 µM resulted in a decrease in frequency of callus induction, which is an agreement with Li et al., (2002) who stated that increasing 2,4-D from 11.3 to 181 µM decreased callus induction in R. hybrida cv. Carefree Beauty.

MS medium containing 1 µM BAP and 0.5 µM NAA showed maximum frequency of regeneration, whereas media containing TDZ showed lesser number of shoot compared to the media containing BAP. Different plant growth regulators play significant roles in the regeneration process of plants in the *in vitro* conditions. TDZ and BAP are the most frequently used cytokinins to induce regeneration, but their effectiveness depends on genotype and other factors (Magyar-Ta´bori et al., 2010). Although, maximum shoot production was observed with medium supplemented with 1 µM BAP and 0.5 µM NAA, this was not significantly different from the result of the treatment with 2.5 µM TDZ and 0.5 µM NAA. In most of the cases, the addition of NAA significantly increased shoot production, it suggest a synergistic/ additive effect of NAA on shoot proliferation. The highest shoot fresh weight was also recorded in the treatment with BAP and NAA. Other reports also showed the synergistic effect of auxins with cytokinins on shoot regeneration and proliferation in medicinal plants (Sudha et al., 1998; Sreekumar et al., 2000; Martin 2002; Beena et al., 2003). Although the number of shoots produced per explant in most treatments was higher (significantly in some cases) than that of the control (Figure 3b), there was no significant difference in the shoot fresh weight of the control when compared to other treatments (1 µM BAP and 0.5 µM NAA, Figure 3c). Shoot fresh weight could have been affected more by the growth of an individual plant rather than the proliferation rate (Bairu et al., 2007; Amoo et al., 2009).

Although the bioactive alkaloid, that is, aconitine, responsible for therapies, are mainly accumulated in the roots of aconitum plant, alkaloid content were also found in the vegetative parts for example, leaves and so on and constantly changes throughout the growth period

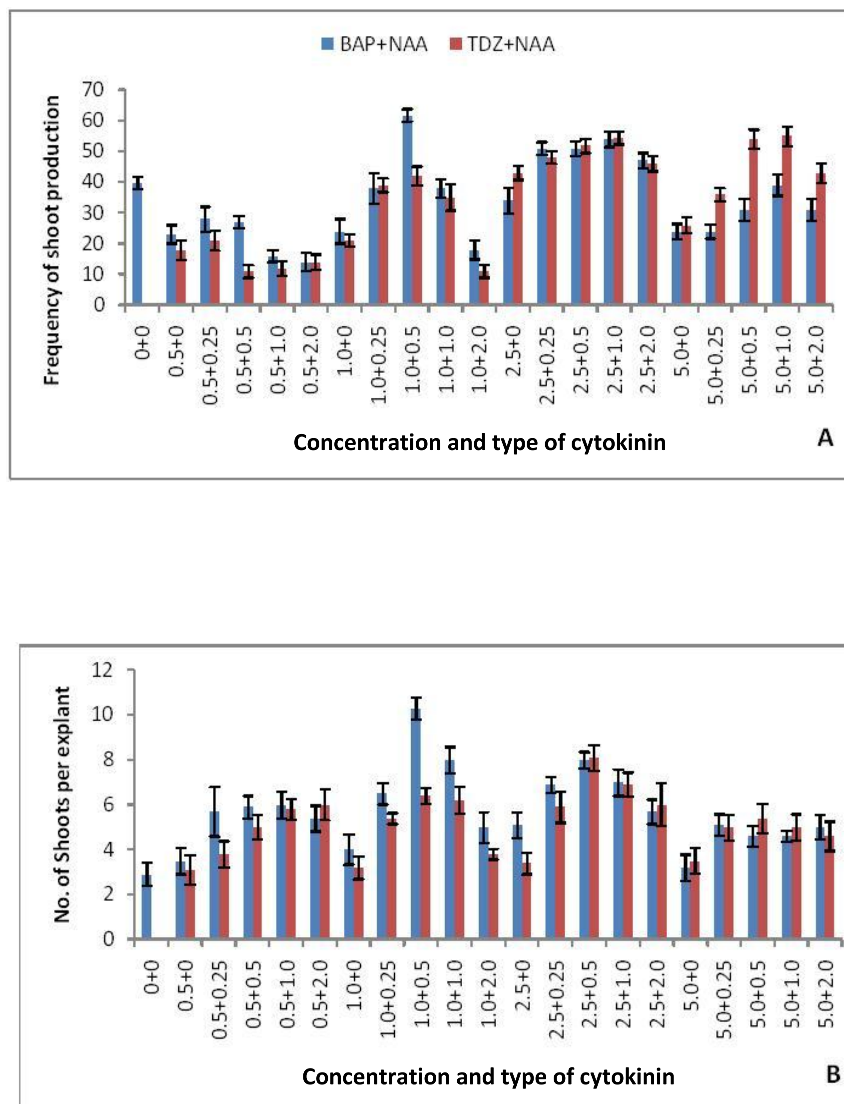


**Figure 3.** Effects of plant growth regulators on shoot proliferation of *A. violaceum* after 8 weeks of culture. A, Frequency (%) of shoot production; B, mean number of shoots regenerated per stem explants; C, mean shoot fresh weight (mg); D, aconitine content in % dry weight.

(Maknickiene 2008; Sinam and Devi, 2011). In the present study, the secondary metabolite analysis has been done in the vegetative part to know the effect of different types and concentration of PGRs. Sinam and Devi (2011) reported that maximum stock of alkaloids in leaves are accumulated before flowering. It is important to mention here that aconitum alkaloid contents can vary with the species, place of origin, time of harvest and most importantly the method and adequacy of processing (Chan et al., 1994).

According to Coenen and Lomax (1997), auxins are known to exhibit synergistic, antagonistic and additive interactions with cytokinins at multiple levels (depending on the plant species and tissue type) in regulating physiological responses. While the level of cytokinins in plants can be regulated by auxins and vice versa, Nordstrom et al. (2004) in their study using transgenic Arabidopsis plants, observed that auxin is a rapid and potent regulator of cytokinin biosynthesis compared to the reverse (where cytokinin regulates auxin synthesis). In addition to controlling fundamental growth and developmental processes in plants, PGRs are also known to regulate the production of plant secondary metabolites in plant tissue culture (Dornenburg and Knorr, 1995). The stimulatory role of cytokinin on the production of secondary metabolite in this plant species might be due to the repression of certain macronutrient transporters, leading to the expression or up-regulation of

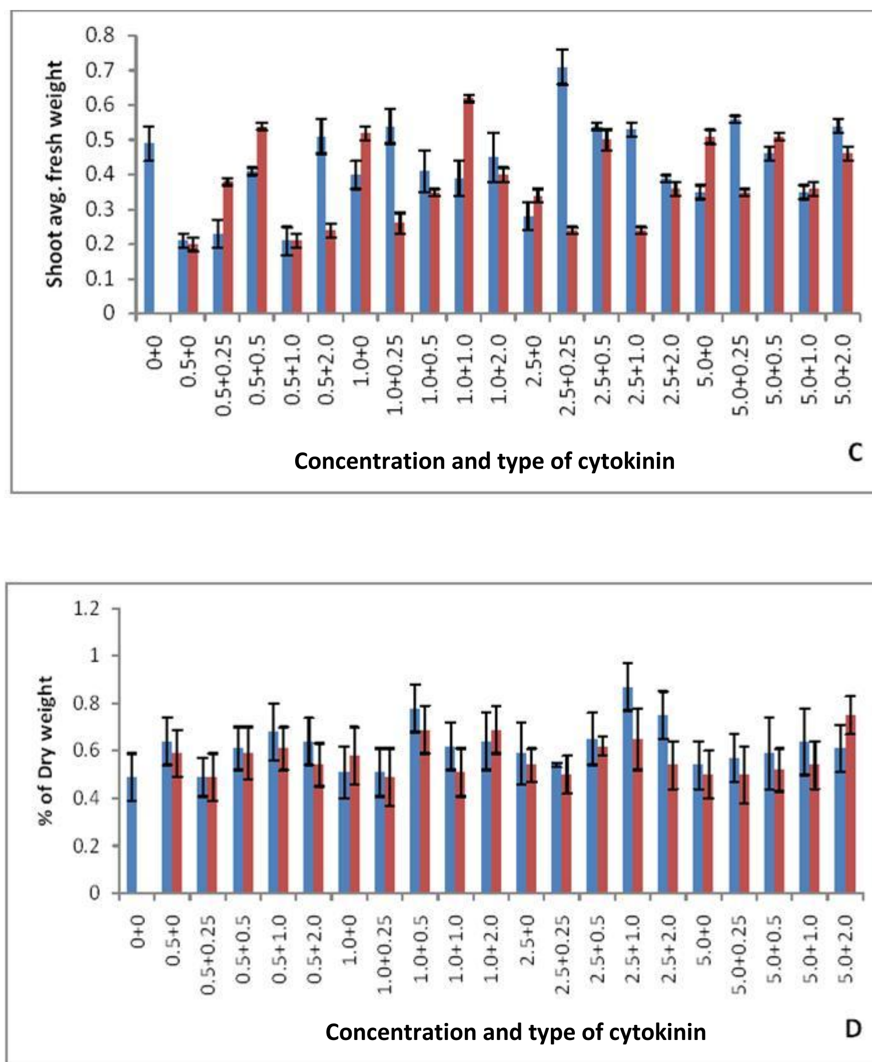


**Figure 3.** Contd.

genes involved in the biosynthetic pathway of secondary metabolites (Sakakibara et al., 2006). According to Sakakibara et al. (2006), cytokinins significantly repress some transporters of macronutrients such as nitrate, ammonium, sulphate and phosphate on one hand, while nitrate on the other hand regulates the expression of genes involved in secondary metabolite pathways. The influence of ytokinins such as BAP, Kn and TDZ as well as auxins such as NAA, 2,4-D and IAA on *in vitro* secondary metabolite production either alone or in combination have been reported by several workers (Meyer and Van Staden 1995; Miura et al., 1998; Luczkiewicz and Cisowski 2001; Coste et al., 2011).

The current study shows the effectiveness of BAP in increasing *in vitro* shoot proliferation and secondary metabolite production in *A. violaceum*, when compared to TDZ. Furthermore, the choice of cytokinin concentration makes a difference in the production level of secondary metabolites. Exogenously applied NAA interacted with cytokinin in a synergistic/additive manner on shoot proliferation, thus increasing the production of regenerated shoots per explant more than three times. On the other hand, low NAA concentrations reduced *in vitro* secondary metabolite production in most cases when compared to treatments with cytokinin only. The current findings highlighted the differential effects of auxin-cytokinin interaction on shoot proliferation and the production of secondary metabolites.

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*Full Length Research Paper*

# Phospholipid fatty acids analysis-fatty acid methyl ester (PLFA-FAME) changes during bioremediation of crude oil contamination soil

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**This study aims to develop certain perspectives based on the principle of on-site remediation of the soil through biological means known as "bioremediation" against soil pollution issues resulting from fuel contamination in our country and to reveal the fatty acid profile in the final soils. The fatty acid profile of the soils was pointed out by testing the activity of three basic bioremediation applications (biological multiplication, biological excitation and the combined application of these two approaches) established in the laboratory condition. Under biological multiplication applications, six of the selected bacterial strains *(Pseudomons aeruginosa, Pseudomonas putida biotype A, Citrobacter amalonaticus-GC subgroup A*, *Acinetobacter genomospecies)* exhibit the highest growth in crude oil environment isolated from oil-contaminated soils of Adana, Batman and Adıyaman, and they also have the highest levels of crude oil degradation. Under biological excitation applications, the organic materials being humic-fulvic acid and, in combined applications, different combinations of bacteria mixture and organic materials were examined as to the amount of crude oil they degrade in an incubation period of 120 days by qualitative hydrocarbon-type analyses. The highest level of oil degradation, being 56%, occurred under biological multiplication applications where the bacteria mixture was applied. Under biological excitation conditions where various organic materials were applied to the contaminated soil, degradation to 18% was observed. In combined applications, oil degradation was achieved to 30%. The most common fatty acids were found to be 15:0 iso, 15:0 anteiso, 16:0, 16:1 w7c, 17:0ai, 18:2w6,9 and 18:1w9c fatty acids detected in both unpolluted and oil-contaminated soils. Determination of high level 18:1w9c fatty acid in oil contaminated and clean soils may indicate the presence of *Pseudomonas* spp. However, fatty acid 15:0 anteiso was determined to be higher in oil-contaminated soils than in unpolluted soils. It may be explained that Gram positive bacteria were predominant in oil-contaminated environment.**

**Key words:** Soil, crude oil, bacteria, bioremediation, phospholipid fatty acids analysis-fatty acid methyl ester (PLFA-FAME).

## INTRODUCTION

It is well known that in soil, number and types of existing microorganisms are affected by biological and physico-chemical events including soil properties such as suitable conditions for microbial decomposition (oxygen, food substance, temperature and pH), microbial decomposition of hydrocarbons, quantity and quality of contaminants and its biological usefulness and particle distribution (Atlas, 1981; Atlas and Bartha, 1992; Steffan et al., 1997; Morgan and Watkinson, 1989; Margesin and Schinner, 1997a). Kapley et al. (1999) demonstrated that fungi can

decompose hydrocarbons, particularly *Emericella nidulans*, *Graphiwn putredinis*, *Eupenicillum javanicum* and *Aspergillus flavipes* are active in the assimilation of aromatic hydrocarbons. They showed that some soil-originated bacteria such as *Pseudomonas* spp. have the capability to decompose some fractions of crude oil. In addition, Jùrgensen et al. (2000) isolated and identified *Enterobacter sakazakii, Bacillus mycoides, Klebsiella oxytaca* and *Acinetobacter calcoaceticus*, from the compost application no. 3 on petroleum-contaminated soils; *Bacillus megaterium, Pseudomonas diminuta, Gluconobacter cerenius* and *Pasteurella caballi* from the compost application No. 1; and *Sphingomonas paucimobilis* and *Sphingobacterium multivorum* and some unknown bacteria from compost application No. 2. Obire and Okudo (1997), Bailey et al. (2002) showed existence of different microbial populations in petroleum-contaminated environment compared with the ones in cleaner environment. These changes in microbial community are the result of food cycle and movement in soil and can be determined by the method of total extractable phospholipid fatty acid.

In spite of the fact that there are many studies conducted on microorganisms which remove particular hydrocarbons or hydrocarbon groups forming structure of petroleum and petroleum products (PPP) in a short time, our knowledge about the role of soil microorganisms in hydrocarbon decomposition is very limited. In the present study, the selected microorganisms and mixture of microorganisms isolated from various soil ecosystems under different environmental conditions were evaluated for elimination of PPP-related contamination in laboratory and field studies in Turkey. For this purpose, an economical and environment-friendly "biological improvement (bioremediation)" approach was taken as a model in the elimination of petroleum and similar organic contaminants. Then fatty acid profiles of the soils were determined by testing the efficiency of three basic bioremediation applications established in the laboratory conditions (bio-augmentation, bio-stimulation and the combined application of these two approaches) in elimination of crude oil based contamination.

## MATERIALS AND METHODS

### Procurement of soil material in which experimental contamination conditions are to be established

Soil material from trial lands of AUZF Research and Application Farm (approximately 40 kg, taken from 0-20 cm depth) were brought to laboratory after sieved through a sieve of 2 mm.

### Contaminating material

Crude oil was procured from Kırıkkale Refinery Premises of Turkish Petroleum Corporation (TPAO).

Composition by weight of hydrocarbon are: alkanes (paraffins) 30%, naphthenes 49% aromatics15%, asphaltics 6%.

### Procurement of the bio-augmentation material (bacteria-bac)

Batman Refinery waste accumulating field, samples were taken from 0-20 cm depth: (waste 1) and samples were taken from 20-40 cm depth: (waste 2). Samples were also taken from Adiyaman TPAO petroleum wells inner station petroleum water accumulating area and "BTC (Bakü - Tiflis - Ceyhan) crude oil loading terminal area in Adana. All samples were kept at +4°C until analyses were made.

### Bio-stimulation material (humic-fulvic- acid)

As bio-stimulation material, soil regulator sold in the market, coded as HFA (humic-fulvic- acid) (K- Humate) was used.

### Preparation of the soil used for bioremediation purposes

The soil used in this study (about 40 kg), was collected one week before the setting of last trial and was kept at room temperature.

### Isolation of bacteria from petroleum-contaminated soils

The following procedures were followed with isolation purposes on the samples taken from i) Batman refinery, refinery waste accumulating area, ii) from Adıyaman TPAO petroleum wells inner station petroleum water accumulating area and iii) from "BTC" Crude oil loading terminal area in Adana. 1% crude oil and Triton-X-100 emulsifier (1:1) were added into 1 L broth medium which include 10 g soil ,1 g $KNO_3$, 0,2 g MgSO4, 0,1 g NaCl, 0,1 g $CaCl_2$ g, 1 g $K_2HPO_4$. Then they were left for incubation at 180 cyc/min at 28°C for 3 days. At the end of the 3rd day, 10 ml was taken out from incubated broth medium and put in a fresh environment again which has the same components (Erdoğan et al., 2011, Rojas-Avelizapa et al., 1999).

### Microbial Identification System (MIS) identification of isolated bacteria

Miller and Berger (1985) carried phospholipid fatty acids analysis (PLFA) analysis made on pure bioremediation bacteria cultures out using MIS. This system is based on the fact that number, variety and quantity as percentage (fatty acid profile) of fatty acids in the cells of microorganisms with same genetics and that they do not change as long as environment conditions remain the same (Şahin, 1997; Şahin et al., 1999; Erdoğan et al., 2011).

### Bioremediation applications

#### *Preparation of experimental contamination conditions*

A 3000 g of soil sample having a moisture content of 50% of its water holding capacity was placed into a plastic pot incubated 25°C for 10 days. After three days of pre-incubation, soil sample was contaminated by applying crude oil of 1% on weight basis (w/w)

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**Abbreviations: PPP,** Petroleum and petroleum products; **HFA,** humic-fulvic- acid; **PLFA,** phospholipid fatty acids analysis; **MIS,** microbial identification system; **TPH,** total petroleum hydrocarbons; **FAME,** fatty acid methyl ester; **GC,** gas chromatographic.

**Table 1.** Experimental design.

| Treatment number | Treatment | Treatment of content |
|---|---|---|
| 1 | *BAC + *N | Biological multiplication |
| 2 | *HFA + *N | Biological excitation |
| 3 | *BAC + *HFA + *N | Combined application (1+2) |
| 4 | C + *N | Control (basic fertilization) |

*BAC, Bacteria mixture; *N, nutrient; *HFA, humic fulvic acid.

homogeneously. Clean soil material (as a control) was left in room conditions for the same duration.

#### *Preparation of bioremediation bacteria mixture cultures*

Six bacterial strains, their petroleum decomposition abilities in liquid culture determined were applied homogeneously to clean and contaminated soils by spraying. The applied bacteria density of each strain was $10^{10}$ CFU/ml. (Erdogan, 2010; Erdoğan et al., 2011).

### Experimental design

Trial was set in 5 kg plastic mouth capped cups (Table 1). Samplings have been done on 1st, 30th, 60th, 90th and 120th day. Total petroleum hydrocarbons (TPH) analyses and cultural count on the1st, 30th, 60th, 90th and 120th day was done for monitoring of crude oil decomposition. PLFA-fatty acid methyl ester (FAME) analysis on the 1st, 30th, 60th, 90th and 120th day using direct extraction method has been done for investigating soil community structure in each sampling. In this way, it allowed determination of "indicator-fatty acid methyl ester" with respect to bioremediation bacteria which is to be used (Erdogan, 2010).

### Determination of efficiency of bioremediation applications

#### *Monitoring crude oil decomposition in soil*

In soil samplings taken on the 1st, 30th, 60th, 90th and 120th day of the trial, petroleum analyses were conducted with ASE device (Dionex ASE 300) in TPAO-Research Central Geochemistry Laboratory (EPA method 3545).

#### *Ascertainment of determinative features of bioremediation bacteria used with PLFA-FAME analysis*

"Indicator-fatty acid methyl ester" of bioremediation bacteria which is to be used was ascertained by making FAME analysis with direct extraction method in order to monitor life status of petroleum decomposing bacteria, which were made resistant to crude oil, in petroleum-free and petroleum contaminated soil.

Essentials of this procedure can be defined as i) Extracted microbial living from soil and having been treated to them with a medium level alkali hydrolysis and break up of their cells; ii)ester binds breaking and fatty acids being separated from lipids and fatty acids; iii) having been transformed into methyl ester form, being analyzed in gas chromatographic (GC) system in quantitative (%) and qualitative respects. Bligh and Dyer (1959), Sasser (1990), Zelles et al. (1992), Frostegard et al. (1993), Bossio et al. (1998), Ibekwe and Kennedy (1998) also used this analysis method in their study.

### Statistical analyses

Results of the study were evaluated with repeated measures variance analysis method with respect to features focused on. Repeated measures were made at the levels of time factor and conducted in three replicates "SPSS 12.0", MSTAT software packages were used for calculations. In addition, correlations between investigated parameters were evaluated using Pearson Correlation Test (Winer et al., 1991; Gürbüz et al., 2003).

## RESULTS

### Species-genus-order-families of isolated bacteria

Results of the isolated bacteria are given collectively in Table 2.

### Changes in total petroleum hydrocarbons (TPH)

TPH values of Pet+BAC+N,Pet+HFA+N and Pet+BAC+HFA+N applications applied on petroleum-contaminated (Pet.) soils for 120-day incubation period are given in Figure 1. When variance analysis table relating to TPH in petroleum-contaminated soils was examined, time-bacteria-HFA triple interaction was found statistically significant ($P<0.05$). When we compare petroleum-contaminated soils in respect of their TPH values, time-dependent reduction is observed in all applications except control. While the biggest reduction was observed in Pet+BAC+N soils, the smallest reduction was observed in control soils. If we are to compare application types with each other, the highest TPH value (7998 mg.kg-1) was determined in soil Pet+BAC+N on the 1st day and the lowest one (4500 mg.kg-1) was determined in soil Pet+BAC+N on the 120th day; the difference between applications was found to be significant statistically at level of $P<0.05$.

Filauro et al. (1998) stated in their study that TPH concentration was decomposed by 48% with bacteria application, Peressuttia et al. (2003) indicated that TPH quantity was reduced by 45.48%. Pokethitiyook et al. (2002) found out that *A. Calcoaceticus* isone among those isolated from a petroleum-contaminated area in Bangok, and three Pseudomonas strains provided high level of decomposition at 0.5% crude oil level.

**Table 2.** Genus-species-order-families of isolated bacteria.

| Bacteria (Genus-species) | Order - Family |
|---|---|
| *Pseudomonas putida* | Pseudomonadales - Pseudomonadaceae |
| *Pseudomonas aeruginosa* | Pseudomonadales - Pseudomonadaceae |
| *Pseudomonas mucidolens* | Pseudomonadales- Pseudomonadaceae |
| *Acinetobacter genomospecies* | Pseudomonadales - Moraxellaceae |
| *Stenotrophomonas maltophilia* | Xanthomonadales - Xanthomonadaceae |
| *Enterobacter hormaechei* | Enterobacteriales - Enterobacteriaceae |
| *Enterobacter sakazakii* | Enterobacteriales - Enterobacteriaceae |
| *Citrobacter amalonaticus* | Enterobacteriales - Enterobacteriaceae |
| *Eschericha coli* | Enterobacteriales - Enterobacteriaceae |
| *Sphingobacterium multivorum* | Sphingobacteriales - Sphingobacteriaceae |
| *Aeromonas caviae* | Aeromonadales - Aeromonadaceae |
| *Paucimonas-lemoignei* | Burkholderiales - Burkholderiaceae |

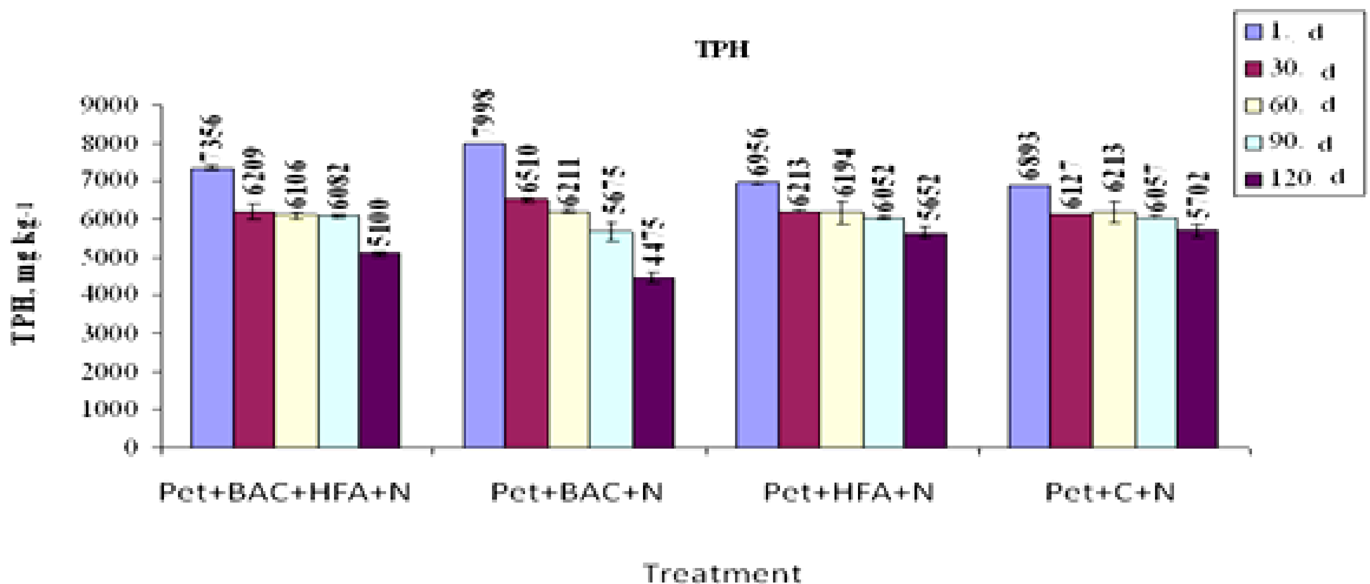


**Figure 1.** TPH values relating to applications Pet+BAC+N, Pet+HFA+N and Pet+BAC+HFA+N applied on petroleum-contaminated soils.

*A. calcoaceticus*, the best among four bacteria, provided decomposition values of 80.76, 68.86 and 65.18% in contaminated soil at different temperatures of 20, 40 and 30°C, respectively.

The data of another study conducted by Ghazali et al. (2004) is very similar and supporting our results. Researchers found out that two different bacterial mixtures isolated from contaminated soil showed similar and high levels of petroleum decomposition under soil-free *in vitro* conditions. They indicated that a bacterial strain isolated from contaminated soil and bacterial mixtures composed of *Pseudomonas* spp and *Bacillus* spp provided a higher level of decomposition tested under conditions of diesel, petroleum and machine oil-contaminated soil for a 60-day incubation process. Rambeloarisoa et al. (1984) found out that a bacterial mixture, which is composed of 8 species belonging to six different bacterial genera, effectively decomposed crude oil.

In another study, Kishore and Ashis (2007) tested *Bacillus subtilis*(DM-04), and *Pseudomonas aeruginosa* (M) isolated from a petroleum-contaminated area at southeastern India under soil-free *in vitro* and contaminated soil conditions, and showed that *P. aerugionasa* (M) was more effective than *B. subtilis* (DM-04) in soil-free environment. After a 120-day incubation period in the petroleum-containing soil experiment, it was observed that while *B. subtilis* DM-04 eliminated 50% of the crude

**Table 3.** Change over time of measurable fatty acids of humic acid and bacteria (Pet+HFA+BAC+N) application under petroleum containing conditions.

| Pet+HFA+BAC+N | Time | | | | |
|---|---|---|---|---|---|
| **PLFA-FAME** | **1st day** | **30th day** | **60th day** | **90th day** | **120th day** |
| 10:0 | 3.36 | 13.3 | - | - | - |
| 11:0 | 4.07 | - | - | - | - |
| 12:0 | 1.88 | - | - | - | - |
| 12:0 iso 3OH | 3.39 | - | - | - | - |
| 12:0 anteiso | 1.22 | 30.64 | - | - | - |
| 13:0 anteiso | 2.43 | 21.68 | - | - | - |
| 15:0 iso | 4.77 | - | - | - | - |
| 15:0 anteiso | 13.35 | 34.38 | 100.00 | - | - |
| 16:0 | 6.23 | - | - | - | - |
| 16:0 anteiso | 11.35 | - | - | - | - |
| 16:0 iso | 7.56 | - | - | - | - |
| 16:0 10-methyl | 5.47 | - | - | - | - |
| 17:1 w5c | 7.5 | - | - | - | - |
| 17:0 anteiso | 6.53 | - | - | - | - |
| 18:0 anteiso | 10.42 | - | - | - | - |
| 18:1 w9c | 3.91 | - | - | - | - |
| 18:3 w6c(6.9.12) | 4.18 | - | - | - | - |

**Table 4.** Change over time of measurable fatty acids of bacteria (Pet+BAC+N) application under petroleum containing conditions.

| Pet+BAC+N | Time | | | | |
|---|---|---|---|---|---|
| **PLFA-FAME** | **1st day** | **30th day** | **60th day** | **90th day** | **120th day** |
| 9:0 3OH | 12.04 | - | - | - | - |
| 10:0 | 11.96 | 28.99 | - | - | - |
| 12:0 anteiso | 12.03 | 71.01 | - | - | - |
| 13:0 anteiso | 10.54 | - | - | - | - |
| 14:0 anteiso | 12.44 | - | - | - | - |
| 15:0 anteiso | 16.93 | - | 100.00 | - | - |
| 18:1 w9c | 17.63 | - | - | - | - |
| 17:0 anteiso | 9.01 | - | - | - | - |
| 18:1 w6.9c | 7.53 | - | - | - | - |
| 18:3 w6c(6.9.12) | 7.29 | - | - | - | - |

oil, *P. aerugionasa* (M) and a bacterial strain isolated from contaminated soil were able to decompose up to 100% of those. Porta et al. (1998) also reported similar results. On the other hand, other researchers indicated that complex hydrocarbons, phenols, phenanthrene and benzopyrene show a high metabolic capability in gram-positive bacteria with respect to decomposing oxidation products (Sextone et al., 1978; Song and Barta, 1990).

## Phospholipid fatty acid methyl ester (FAME)

Evaluation of soil PLFA-FAME analysis is shown in Table 3 to 6 and the number of indicator fatty acid, which could be measured at different times, was different in soil at PLFA-FAME analysis results.

Based on this information, we can see that the most intensive fatty acid in all applications was on the 1st day and it reduced over time; and finally, on the 90th and 120th day fatty acid could not be found (Tables 3 to 6). While in Pet+HFA+BAC+N application, at the beginning of the incubation (1st day), the number of PLFA was 17, it reduced to 4 on the 30th day and to 1 on the 60th day, and later no fatty acid could be found (Table 3). The number of PLFA-FAME in this application was more than that of other applications. This could be due to the

**Table 5.** Change over time of measurable fatty acids of humic acid (Pet+HFA+N) application under petroleum containing conditions.

| Pet+HFA+N | Time | | | | |
|---|---|---|---|---|---|
| **PLFA-FAME** | **1st day** | **30th day** | **60th day** | **90th day** | **120th day** |
| 12:0 anteiso | 20.21 | 24.88 | - | - | - |
| 13:0 anteiso | 18.42 | 16.55 | - | - | - |
| 14:0 anteiso | 21.65 | 8.99 | - | - | - |
| 15:0 anteiso | 22.67 | 22.83 | 100.00 | - | - |
| 16:0 anteiso | 23.53 | 22.54 | - | - | - |

**Table 6.** Change over time of measurable fatty acids of control (Pet+C+N) application under petroleum containing conditions.

| Pet+C+N | Time | | | | |
|---|---|---|---|---|---|
| **PLFA-FAME** | **1st day** | **30th day** | **60th day** | **90th day** | **120th day** |
| 11:0 anteiso | - | 21.59 | - | - | - |
| 12:0 anteiso | - | 29.57 | - | - | - |
| 13:0 anteiso | - | 21.03 | - | - | - |
| 15:0 anteiso | 21.58 | 27.82 | 100.00 | - | - |
| 16:0 anteiso | 20.9 | - | - | - | - |
| 17:0 anteiso | 11.8 | - | - | - | - |
| 18:2 w6.9c | 16.3 | - | - | - | - |
| 18:3 w6c(6.9.12) | 10.61 | - | - | - | - |
| 19:1 w6.9c | 18.80 | - | - | - | - |

addition of bacteria. This quantity fell over time and after the 90th day no PLFA-FAME could be found. While in Pet+BAC+N application, at the beginning of the incubation (1st day), the number of FAME was 10, it reduced to 2 on the 30th day and to 1 on the 60th day, and later no fatty acid could be found (Table 4).

In this application as well, quantity of PLFA-FAME was higher on the 1st day than that of other applications due to the addition of bacteria. While in Pet+HFA+N application, at the beginning of the incubation (1st day), the number of FAME was 5, on the 30th day number of fatty acid was same, the 60th day it reduced to 1, and later no fatty acid could be found (Table 5). While in Pet+C+N application, at the beginning of the incubation (1st day), the number of FAME was 6, it reduced to 4 on the 30th day and to 1 on the 60th day, and later no fatty acid could be found (Table 6).

## DISCUSSION

One of the basic intentions of this study is to determine the microorganisms which can eliminate contamination in soil stemming from petroleum and that kind of materials, and to evaluate their efficiency in laboratory conditions. Applications known as bioremediation are based on use of microorganisms, which have different levels of hydrocarbon decomposing capability, in petroleum-contaminated environments. The most important measure that is taken as basis in revealing bioremediation potential of applications used (bio-augmentation, bio-stimulation, bio-augmentation + bio-stimulation) is soil TPH analysis results based on total hydrocarbons in soil. The highest decrease in total petroleum hydrocarbon rate over time among applications was found in bacteria (Pet+BAC+N) application, which is followed by bacteria + humic fulvic acid (Pet+BAC+HFA+N) application and the lowest decrease was found in the control application. When we look at the 1st and 120th days of the study with respect to TPH, bioremediation process proceeded fastest in bacteria (Pet+BAC+N) application by 56%, which is followed by bacteria + humic acid (Pet+BAC+HFA+N) application by 30%, only humic fulvic acid (Pet+HFA+N) application by 18% and control application by 17%. The data showed that mixture of bacterial strains (*P. aeruginosa, Pseudomonas putida* biotype A, *Citrobacter-amalonaticus-GC subgroup* A, *Acinetobacter-genomospecies*) gave the best result. In the case of decrease level in TPH values in bacteria (Pet+BAC+N) application, the fastest degradation was measured between 1-30 days and 90-120 days. It is seen that between 30-90 days of incubation, there is a very little change. This result indicated that petroleum decomposition is not continuous but has some inactive periods. It was observed that other applications also have the same inactive phase. Different PLFA bio-indicators were used to gather information about many

members of soil micro flora such as bacteria, fungi, algae, gram-negative bacteria, gram-positive bacteria, sphingomonas, actinomycetes and sulphate-reducing bacteria. It was indicated that these agent materials provide clues about lipid synthesis that soil microorganisms make in connection with many environmental events (White et al., 1996a, b; Venosa et al., 2000). Olsson and Persson (1999) reported that 18:1ω9c is also an indicator representing *Pseudomonas* spp. bacteria. Since fatty acid 18:1w9c showed high values in bacteria (Pet+BAC+N ve Pet+BAC+HFA+N) applications we conducted in clean and contaminated soils, we can put emphasis on that it is a specific fatty acid of *Pseudomonas* spp. bacteria. 15:0 iso is an indicator for gram-positive and sulphur bacteria (Olsson and Persson, 1999). It showed reduction over time in all applications. Other researchers indicated that for (i15:0 and a15:0) gram-positive bacteria, their biomarkers increase (Ringelberg et al., 2008). It was determined that the level of the 15:0 anteiso fatty acid is higher in petroleum-contaminated soils than in unpolluted soils. We may emphasize that the existence of gram-positive bacteria increases in petroleum-contaminated environment. It was indicated that saturated fatty acid 16:0 is an indicator which can be found in all bacteria generally (Pelz et al., 2001; Keinanen et al., 2003), however, Kneif et al. (2006) stated that PLFA 16:0 is only specific to methane-oxidizing bacteria (metanotroph) as a source of carbon and energy. Some researchers indicated that biomarkers of fatty acids n16:1w7c, n18:1w9c and n18:1w7c increase for gram-negative bacteria (Ringelberg et al., 2008). When investigated in respect of 18:1ω9c concentrations ascertained in our study, the common point is that it is a fatty acid occurring in bacteria (Pet+BAC+N) applications, and this PLFA agent had a high value at the beginning of the incubation while at the end of it, it decreased and on the 120th day it could not be found. If 18:1ω9c is an indicator of species *Pseudomonas* spp. as it is claimed by Olsson and Persson (1999), then we can relate the reduction of this indicator fatty acid concentration over time to the decrease of hydrocarbon sources in the environment. According to the study of Parker and Taylor (1983) and Guckert et al. (1985) 18:1ω7c and 18:1ω9c are agents specific to aerobic bacteria and also Fierer et al. (2003) grouped these fatty acids as gram-negative bacteria indicator. Bundy et al. (2002) related the fatty acids 18:1w9 and 17:1w8, which are extracted from petroleum-contaminated soil, to gram-positive bacteria. Fatty acid 17:0ai was observed more in number in clean soil than petroleum-contaminated soil.

PLFA agents (fatty acids) determined are biomarker for gram-negative bacteria and the fact that this PLFA agent has a high value at the beginning of the incubation and it decreases at the end of the incubation is related to the reduction in hydrocarbon sources in the environment. In clean and petroleum-contaminated soils, mostly fatty acids 15:0 iso, 15:0 anteiso, 16:0, 16:1 w7c, 17:0ai, 18:2w6, 9, and 18:1w9c were detected. Because the fatty acid 18:1w9c showed high levels, we may state that it is a fatty acid specific to the bacteria *Pseudomonas* spp. We could not yet reach a clear answer about how to use bioremediation applications, a new subject in Turkey, for the elimination of contamination, despite the scientific efforts made in our research institutions. The present study will cast light on the solution of a potential problem in Turkey. In this regard, in medium-level lime, light alkali, 1% (w/w) petroleum-contaminated soils, the highest petroleum decomposition, that is, 56%, occurs under bio-augmentation applications in which bacteria mixture is applied and what should be kept in mind is that the applied augmentation material consists of local bacteria.

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*Full Length Research Paper*

# Larvicidal activity of extracellular secondary metabolites from a *Stereum* species Hill ex Pers. (JO5289) against the dengue fever mosquito, *Aedes aegypti* (Linn) (Diptera: Culicidae)

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**The main objective of this investigation was to find mosquito larvicidal secondary metabolites from a basidiomycete – *Stereum* species (JO5289) – against *Aedes aegypti*. The *Stereum* species (JO5289) was collected in July 2005 from undisturbed habitat in Londiani forest in Rift Valley province, Kenya. Extracellular crude extracts from *Stereum* species (JO5289) produced strong activity against *A. aegypti* larvae. Purification of the crude extracts targeting larvicidal activity using chromatography gave three active compounds namely; tyrosol, 3-methoxy-5-methyl-1,2-benzenediol and 2-hydroxy-4-(4-hydroxy-chroman-7yl) but-3-enal. The chemical structures of the compounds were determined using the nuclear magnetic resonance (NMR) spectral data and comparison with literature values. When tested for larvicidal activity, the $LC_{50}$ for the three compounds were 26.7, 17.3 and 14.5 ppm, respectively, while $LC_{90}$ were 85.3, 83.5 and 82.9 ppm, respectively, after 24 h of exposure. These compounds have been produced from cultures of a *Stereum* species and reported to have mosquito larvicidal activity for the first time.**

**Key words:** Basidiomycete, *Stereum* species, *Aedes aegypti*, extracellular metabolites and larvicidal.

## INTRODUCTION

Mosquitoes are the major vector for diseases such as malaria, filariasis, dengue fever, Japanese encephalitis and several other diseases globally (Rahuman et al., 2009; Borah et al., 2010). Indeed, the recrudescence of these diseases is due to high number of breeding places and the increasing resistance of mosquitoes to the used commercial insecticides (Chowdhury and Chandra, 2008). The most successful method of minimizing the incidence of mosquito-borne diseases is to eradicate and control the mosquito vectors (Rozendaal, 1997). In the past few decades, synthetic insecticides were used as mosquito control agents but have produced a negative impact on environment, ill effect on non-target organisms and most mosquito species becoming physiologically resistant (Arivoli et al., 2011). Moreover the repeated use of synthetic insecticides has disrupted natural biological systems and led to resurgences in mosquito populations with associated insect resistance (Prabhu et al., 2011).

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**Abbreviations: PDA,** Potato dextrose agar; **TLC,** thin layer chromatography; **NMR,** nuclear magnetic resonance; **LDL,** low density lipoproteins.

These factors have necessitated a search for eco-friendly, biodegradable and target specific insecticides against the mosquitoes. In recent years, the emphasis to control the mosquito population has shifted steadily from the use of conventional chemicals towards more specific and environmentally friendly materials, which are generally of botanical origin (Neetu et al., 2007; Arivoli et al., 2011).

Fungi are extremely diverse group of heterotrophic organisms that are exploited by humans for various biotechnology applications. The wide range of biologically active secondary metabolites from basidiomycetes has been one of the most attractive groups of natural products studied (Getha et al., 2009). In this study a *Stereum* species belonging to the fungal division of basidiomycetes was studied for larvicidal compounds from its submerged cultures. *Stereum* is type genus of the Stereaceae family of fungi, in the Russulales order. Common names for species of this genus include leaf fungus, wax fungus, and shelf fungus. *Stereum* contains 27 species that have a widespread distribution (Kirk et al., 2008). *Stereum* species are found to live on all kinds of deadwood or hardwood or dead leaves, hence are saprophytic basidiomycetes. Sometimes they are also found on living leaves. *Stereum* species are wood decay fungi that do not have tubes. They are simply small bracket-shaped membranes appearing on dead wood. The underside of the membrane contains spores but no ornament, that is, gills, of any kind. Like most members in the family, *Stereum* lack clamp connection and have amyloid spores.

*Stereum* species are good producers of secondary metabolites in submerged cultures according to various studies that have been reported (Mantle and Mellows, 1973; Mellows et al., 1973; Nair and Anchel, 1975; Ayer and Saeedi-Ghomi, 1981; Sun et al., 2011; Isaka et al., 2012). Several biologically active compounds have also been reported from *Stereum* species. *Stereum hirsutum* is one of several fungi involved in a grapevine disease called esca. From the culture medium of this fungus, four new acetylenic compounds have been isolated and identified with structural elucidation and biological activity reported (Dubin et al., 2000). Omolo et al. (2002) isolated four new pentasubstituted phenolic compounds, named hericenols A, B, C, and D, 6-hydroxymethyl-2,2-dimethylchroman-4-one and the known erinapyrone C from extracts of submerged cultures of a *Stereum* species. Hericenol A showed weak antimicrobial activity while hericenol C was weakly cytotoxic. From the fungus *S. hirsutum*, two new compounds together with two known epidioxysterols have been isolated and identified. Epidioxysterols have been shown to possess a significant activity against *Mycobacterium tuberculosis* (Cateni et al., 2007). Li et al. (2008) isolated five cadinane sesquiterpenoids, named stereumin A, B, C, D and E from the chloroform extract of the culture broth of a *Stereum* species CCTCC AF 207024. The five cadinane sesquiterpenoids showed nematicidal activities against the nematode *Panagrellus redivivus* at 400 mg/l.

From the reported studies on secondary metabolites of *Stereum* species, it is evident that this genus of basidiomycetes is a very rich producer of compounds with interesting biological activities. It is on this basis that we screened *Stereum* species (JO5289) for larvicidal compounds against larvae of *Aedes aegypti*.

## MATERIALS AND METHODS

### General preparations and analytical procedures

Hands, working benches, blades and the general working environment were sterilized using 70% ethanol while the liquid media was heat sterilized using an autoclave (Danfoss 59407-3 NO. 375). The liquid media was constituted by dissolving 1% malt media, 0.4% of glucose and 0.4% of yeast extract in tap water. The pH of the media was adjusted to 5.5, and then dispensed in 250 ml Erlenmeyer flasks. The flasks were corked with cotton wool plugs and finally wrapped with aluminium foil. The set up was sterilized at a temperature of 121°C and pressure of 1.5 bars for about 15 min. Potato dextrose agar (PDA) solid media were prepared by autoclaving 39.0 g PDA in 1 l distilled water and then cooled to 45°C. This was then dispensed as 15 ml per sterile Petri dishes in sterile lamina flow hood. The preserved mycelia material on agar slants was then transferred onto PDA plates. These solid cultures were allowed to grow for two weeks at IBRL at ambient conditions.

Analytical thin layer chromatography (TLC) was performed with Macherey–Nagel pre-coated silica gel 60 $F_{254}$ plates (ALUGRAM ® SIL G/$UV_{254}$ 0.25 mm). Column chromatography was packed with silica gel 60 (0.063 – 0.2 mm/70-230 mesh). The developed TLC plate was viewed under dual fixed wavelength UV lamp (λ = 254 nm and 365 nm) and the spots visualised by spraying with freshly prepared *p*-anisaldehyde solution, heated to 115°C. The larvicidal experiments were set up in glass beakers. The crude extract and the purified compound were kept under 4°C except when undergoing analysis. The purified compounds were dissolved in deuterated chloroform $CDCl_3$). $^1H$ NMR and $^{13}C$ NMR spectra were recorded with 300 MHz Bruker AVANCE NMR spectrometers. COSY, HMBC, HSQC and NOESY were acquired using the standard Bruker software.

### Sample collection and preservation

Pieces of dead wood colonised by *Stereum* species as evidenced by the fruiting body was collected in July 2005 from a natural habitat in Londiani Forest, Kenya. The collected material was assigned an accession number of JO5259. Upon collection pure culture was prepared by trapping spores from the underside (the hymemium) of a sterile piece of the fruiting body. The culture was preserved as agar slants and the corresponding herbarium material are kept in the Integrated Biotechnology Research Laboratory (IBRL) at Egerton University.

### Fermentation of the Stereum species (JO5289)

The sterilized liquid media was used to culture the *Stereum* species (JO5289). Pieces of agar plugs (1 x 1 cm) from a well grown PDA plate were cut and then introduced aseptically into 250 ml liquid media. These were allowed to grow at room temperature and upon assuming steady growth rate were used to inoculate a 20 l fermentor. *Stereum* species (JO5289) was fermented in a 100 l

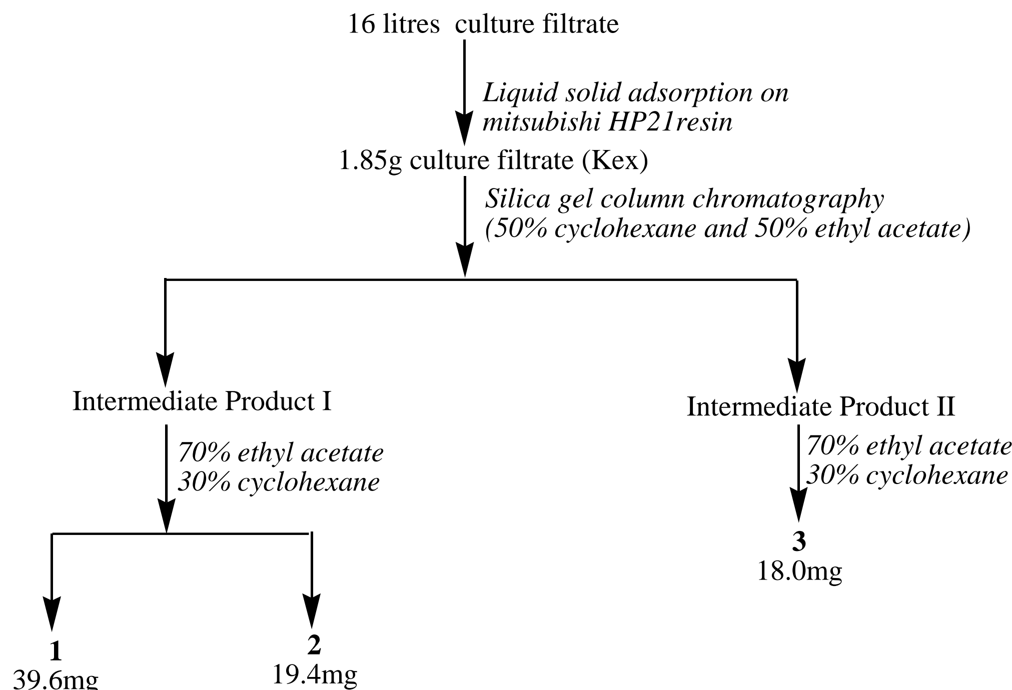


**Figure 1.** Purification scheme for compounds 1, 2 and 3 from *Stereum* species (JO5289).

fermentor using fermentor type U100 (Braun, Melsungen) with online system control Micro-MFCS 3.2 monitoring $O_2$-uptake, $CO_2$-production and oxygen partial pressure in the medium. The medium was aerated at 15 l per minute and stirred at 120 rpm and at 24°C for 6 days.

The pH value of the culture broth during the fermentation period was determined. This was done by withdrawing samples at regular intervals and pH measured using a pH meter (CG 825, Schott, Hofheim). The growth was also closely monitored and evaluated daily to check for glucose depletion using glucose testing stripes. Immediately glucose levels in the culture were depleted, the growth was stopped and the mycelia were separated from culture filtrate by vacuum filtration. From culture filtrate, crude extracts for extracellular secondary metabolites was prepared.

### Preparation of crude extracts from culture filtrate

The culture filtrate was passed through a resin (Mitsubishi HP21 DIAION resin) packed in a column thrice. Once all the culture filtrate passed through the resin, the trapped secondary metabolites were eluted with 3 l acetone followed by 2 l of methanol. The eluents were collected and concentrated into a residual aqueous remain, which was further extracted five times with ethyl acetate. The combined ethyl acetate solution extract was dried using anhydrous sodium sulphate and concentrated using rotary evaporator under reduced pressure. The dried crude extract was weighed and kept at 4°C awaiting further analysis.

### Purification and identification of larvicidal compounds

The crude extract was tested for larvicidal activity against *A. aegypti* before fractionation and purification of the active compounds using repeated silica gel column chromatography. The solvents used were cyclohexane, ethyl acetate and methanol in ratios of increasing polarity. The eluents from the column were pooled into five intermediate fractions, concentrated under reduced pressure and tested for larvicidal activity to track the active compounds. The active intermediate fractions were further subjected to repeated column chromatography until pure compounds were obtained (Figure 1).

Structures of purified larvicidal compounds (Figure 2) were elucidated using nuclear magnetic resonance (NMR) spectroscopic experiments. NMR experiments were performed on 300 MHz Bruker AVANCE NMR spectrometer. The spectra were recorded in deuterated chloroform ($CDCl_3$) and the chemical shifts were recorded in parts per million (ppm) relative to the solvent signals. The deuterated chloroform was referenced according to the central line at δ 7.260 in the $^1$H NMR spectrum and at δ 77.23 in the $^{13}$C NMR. The purified compounds were dissolved in deuterated chloroform ($CDCl_3$) in a clean vial. The solution was then transferred to an NMR tube and was placed in the probe for analysis. The same sample was used to obtain the spectra ($^1$H, $^{13}$C, DEPT, NOESY, HMBC and COSY) data for the compound. Data was acquired from the NMR machine as computer print out. From the spectroscopy experiments, structures were proposed based on the interpretation of the spectra and reported literature values.

### Mosquito larvicidal assay

Eggs of the *A. aegypti* mosquitoes were hatched by submerging them in de-chlorinated tap water at a temperature in the range 25–27°C, as described by the standard WHO protocol (1973). Both the crude extracts and the purified samples were subjected to mosquito larvicidal activity test against late 3$^{rd}$ and early 4$^{th}$ in star larvae of *A. aegypti*. The larvicidal tests were carried out in the Integrated Biotechnology Research Laboratory (IBRL), Egerton University. *A. aegypti* was used as a serotome laboratory specimen given the ease of rearing and the stability of its larval stage. The data generated can be extended to other species of mosquito when the iden-

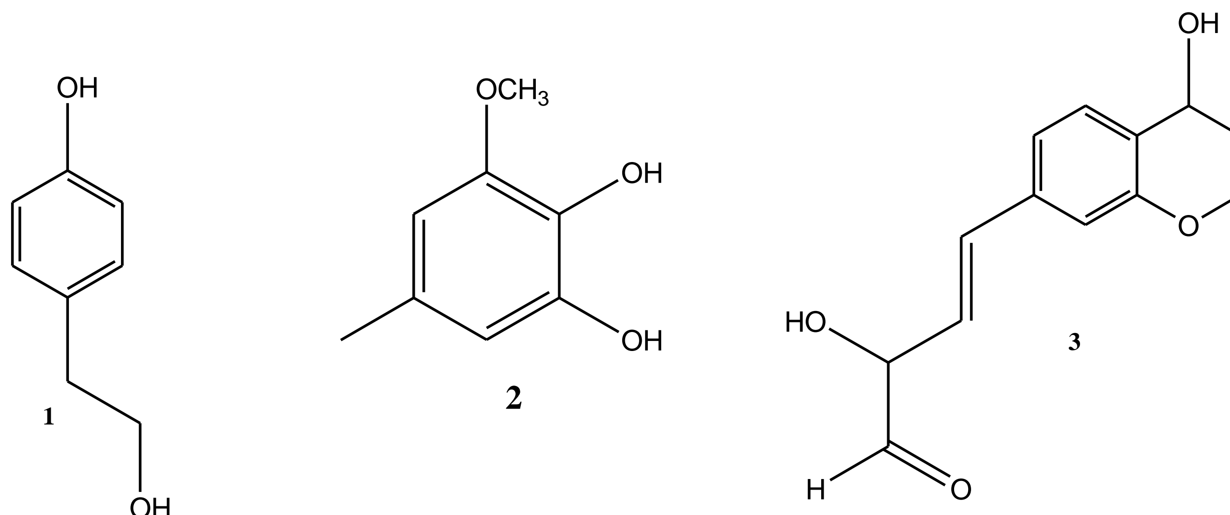


**Figure 2.** Chemical structures for purified compounds 1, 2 and 3.

tified compounds are to be developed for field larvicidal assays.

Standard methods for assaying larvicidal activity were conducted according to the WHO manual (WHO, 2005). Bioassays were carried out in five replicates using 10 larvae for each assay. Varying range of concentrations (2, 5, 10, 20, 50 and 100 ppm) of the crude extracts, fractions and purified compounds were tested against the late third and/or early fourth instar larvae of *A. aegypti*. Aqueous solutions of methanol were employed as the positive control experiments. The larvae were placed in test plastic pots containing 4 ml of the test solution. The number of dead larvae was determined after 2, 4, 8, and 24 h to monitor the larval mortality.

#### Data analysis

The analysis of larvicidal assay data was carried out using regression analysis. The $LC_{50}$ and $LC_{90}$ values which were the concentration values for killing 50 and 90% larvae at 8 and 24 h, respectively, were calculated. Statistical analysis of the data was performed according to the method of Lentner et al. (1982). $LC_{50}$ and $LC_{90}$ were calculated using multiple linear regression and data are expressed as mean±standard deviation (Finney, 1971).

## RESULTS

### Taxonomy

The culture grew in the PDA solid medium as moist brown mycelium that covered a diameter of 5 cm in days. The hyphal strands were found to be septate but with no pronounced clamp connections. It was a resupinate fungus with encrusted hyphae (2.5 μm wide) and quite characteristic closely packed septate cystidia. It also had 4-spored basidia (5 μm wide and 12-15 μm long) producing abundant pin-head basidiospores which were non-amyloid and globose to subglobose (2.5-3 μm wide by 5-6 μm). The observations fitted descriptions for *Stereum* species by Reid (1965).

### Growth and crude extract yield

The *Stereum* species (JO5289) was grown in defined liquid nutrient media (10 g/L malt extract, 0.4 g/L yeast extract and 0.4 g/L glucose) and produced extra cellular secondary metabolites that were harvested as culture filtrate. The yield from 20 l fermentation led to production 16 l of culture filtrate, which further led to 1.85 g of brown semi-solid crude extract after concentration using a rotary evaporator. This translates to 0.09 g per litre of culture filtrate. The crude extract from culture filtrate showed a stronger larvicidal activity than the mycelia crude extract. Hence the culture filtrate crude extract was investigated further while the mycelia crude extract was discarded because it did not have any larvicidal activity in an initial preliminary screening. The larvicidal activity of the culture filtrate crude extract is given in Table 1 as percent mortality when the assay was evaluated after 2, 4, 8 and 24 h.

### Purification of larvicidal compounds

Glass column of 2.5 × 25 cm was packed with 40 g of silica gel (60–120 mesh, Macharey Nagel, Germany) as a slurry using cyclohexane. After testing the crude extract, the remaining amount (1.5 g) was prepared into slurry with 1 g of silica gel. It was loaded in the column and eluted with increasing polarities of organic solvents: cyclohexane:ethyl acetate (9:1), cyclohexane:ethyl acetate (7:3), cyclohexane:ethyl acetate (1:1), cyclohexane:ethyl acetate (3:7) and ethyl acetate (100%) and five fractions were collected, respectively. The total solvent mixture volume was 500 ml and the eluent fraction collected was approximately 500 ml. The fractions were concentrated and tested for larvicidal activity and third fraction that eluted with 1:1 solvent ratio showed the strongest

**Table 1.** Larvicidal activities (% mortality) and lethal concentrations (ppm) of the culture filtrate crude extract (Kex).

| Concentration (ppm) | 2 h | 4 h | 8 h | 24 h |
|---|---|---|---|---|
| 0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| 2 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| 5 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| 10 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 20.2±0.5 |
| 20 | 0.0±0.0 | 0.0±0.0 | 21.0±1.3 | 80.0±1.0 |
| 50 | 0.0±0.0 | 20.2±1.4 | 41.3±1.5 | 100.0±0.0 |
| 100 | 0.0±0.0 | 31.0±1.5 | 51.6±1.5 | 100.0±0.0 |
| Lethal concentrations (ppm) | | | | |
| $LC_{50}$ | - | - | 100.0±1.2 | 35.9±0.7 |
| $LC_{90}$ | - | - | 185.5±1.6 | 87.2±0.9 |

**Table 2.** Larvicidal activities (% mortality) for tyrosol (1) during the assay period.

| Concentration (ppm) | 2 h | 4 h | 8 h | 24 h |
|---|---|---|---|---|
| 0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| 2 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 20.0±1.2 |
| 5 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 40.7±1.2 |
| 10 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 52.0±1.0 |
| 20 | 0.0±0.0 | 0.0±0.0 | 21.0±0.9 | 59.5±0.9 |
| 50 | 0.0±0.0 | 10.0±1.3 | 30.0±1.1 | 80.0±1.1 |
| 100 | 0.0±0.0 | 10.0±1.3 | 40.1±1.2 | 100±0.0 |

activity and it was further subjected to silica gel chromatography. The repeated silica gel was eluted with the same solvent system which led to the larvicidal compounds eluting with the solvent ration 30% cyclohexane and 70% ethyl acetate to afford two intermediate fractions I and II (Figure 1). From intermediate fraction I, two pure compounds were purified tyrosol (1) (39.6 mg) and 3-methoxy-5-methyl-1,2-benzenediol (2) (19.4 mg) while intermediate fraction II afforded one compound identified as 2-hydroxy-4-(4-hydroxy-chroman-7yl) but-3-enal (3) (18.0 mg). The compound was identified by interpretation of the $^1H$ and $^{13}C$ NMR spectral data and comparison with reported literature values.

## Mosquito larvicidal results

The larvicidal activity of the crude extract and the pure compounds at different concentrations (2, 5, 10, 20, 50 and 100 ppm) was evaluated against the late 3$^{rd}$ instar and early 4$^{th}$ instar larvae of *A. aegypti* after 2, 4, 8 and 24 h of exposure. The results are given in Tables 1 to 4 while the corresponding $LC_{50}$ and $LC_{90}$ values are presented in Table 5. The results of the regression revealed that the mortality rate was positively correlated with the concentration range used, at 8 and 24 h of exposure for both the crude extracts and purified compounds. The regression coefficients in all the cases were close to unity (1.0).

The crude extract gave significant activity at 24 h exposure (Table 1), which showed $LC_{50}$ and $LC_{90}$ of 35.9 and 87.2 ppm, respectively (Table 5). These values of mortality rates at 24 h were more pronounced than at 8 h exposure when the $LC_{50}$ and $LC_{90}$ were 100.0 and 185 ppm, respectively. The results were significant at $p < 0.05$. However, at 2 and 4 h of exposure, there was no significant mortality rate observed for the crude extracts and the pure compounds. Therefore the results were not regressed and there were no corresponding $LC_{50}$ and $LC_{90}$ values. The pure compounds showed varied larvicidal efficacy with all of them showing significant activity at 8 and 24 h of exposure. Mosquito larvicidal efficacies of the purified compounds were relatively higher than that for the crude extract. This suggested that the purification process produced more active compounds, an indicator that bioactivity guided fractionation and purification led to the active chemical compounds responsible for the larvicidal activity that was in the extract.

Tyrosol (1) showed a strong larvicidal activity at 24 h for the concentration ranges tested (Table 2) with $LC_{50}$ and $LC_{90}$ of 26.7 and 85.3, respectively (Table 5). At 8 h of exposure, compound (2) showed relatively weaker larvi-

**Table 3.** Larvicidal activities (% mortality) for 3-methoxy-5-methyl-1,2-benzenediol (2) during the assay.

| Concentration (ppm) | 2 h | 4 h | 8 h | 24 h |
|---|---|---|---|---|
| 0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| 2 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 20.0±0.8 |
| 5 | 0.0±0.0 | 0.0±0.0 | 10.0±0.9 | 30.0±0.9 |
| 10 | 0.0±0.0 | 0.0±0.0 | 20.0±0.8 | 50.0±1.1 |
| 20 | 0.0±0.0 | 20.0±1.1 | 50.0±1.0 | 90.0±1.2 |
| 50 | 10± | 40.0±1.0 | 60.0±1.2 | 100.0±0.0 |
| 100 | 10± | 50.0±1.3 | 70.0±1.2 | 100.0±0.0 |

**Table 4.** Larvicidal activities (% mortality) for 2-hydroxy-4-(4-hydroxy-chroman-7yl) but-3-enal (3) during the assay.

| Concentration (ppm) | 2 h | 4 h | 8 h | 24 h |
|---|---|---|---|---|
| 0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| 2 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 20.0±1.5 |
| 5 | 0.0±0.0 | 0.0±0.0 | 20.0±0.2 | 40.0±1.2 |
| 10 | 0.0±0.0 | 0.0±0.0 | 30.0±1.2 | 60.0±0.9 |
| 20 | 0.0±0.0 | 20.0±0.8 | 60.0±0.4 | 80.0±1.1 |
| 50 | 10.0±0.0 | 40.0±0.9 | 70.0±0.4 | 90.0±1.4 |
| 100 | 10.0±0.0 | 50.0±1.2 | 90.0±0.9 | 100±0.0 |

**Table 5.** Lethal concentrations: $LC_{50}$ and $LC_{90}$ (ppm) for the purified compounds 1, 2 and 3 after 8 and 24 h.

| Compound name | $LC_{50}$ (ppm) | | $LC_{90}$ (ppm) | |
|---|---|---|---|---|
| | 8 h | 24 h | 8 h | 24 h |
| Tyrosol (1) | 127.0±1.4 | 26.7±0.2 | 235.0±1.8 | 85.3±0.6 |
| 3-Methoxy-5-methyl-1,2-benzenediol (2) | 63.4±0.6 | 17.3±0.2 | 136.4±1.6 | 83.5±0.7 |
| 2-Hydroxy-4-(4-hydroxy-chroman-7yl) but-3-enal (3) | 43.0±0.2 | 14.5±0.2 | 100.0±1.3 | 82.9±0.6 |

cidal activity, with $LC_{50}$ and $LC_{90}$ values of 235.0 and 127.0 ppm. Compound (2) (3-methoxy-5-methyl-1,2-benzenediol) showed larvicidal activity at 8 and 24 h of exposure that could the correlated to the concentration range used (Table 3). This gave $LC_{50}$ and $LC_{90}$ for (2) of 136.4 and 63.4 ppm, respectively, at 8 h of exposure. While at 24 h of exposure, it had $LC_{50}$ and $LC_{90}$ of 83.5 and 17.3 ppm, respectively. Compound (3) (2-hydroxy-4-(4-hydroxy-chroman-7yl) but-3-enal) also showed larvicidal activity at 8 and 24 h of exposure that correlated with the concentration ranges used in the bioassay (Table 3). The $LC_{50}$ and $LC_{90}$ for (3) were 100.0 and 43.0 ppm at 8 h of exposure, respectively while at 24 h of exposure the values were 82.9 and 14.5 ppm, respectively. At 24 hours of exposure it was clear that the order of activity was 3 > 2 > 1 > crude extract. This is evident when the $LC_{50}$ and $LC_{90}$ values are compared, but at 8 h of exposure the crude extract appear to be stronger than tyrosol (1).

## Structure elucidation

The crude extract was closely analysed using TLC and further purified using column chromatography. From these only three compounds tyrosol (1), 3-methoxy-5-methyl-1,2-benzenediol (2) and 2-hydroxy-4-(4-hydroxy-chroman-7yl) but-3-enal (3). The data obtained from NMR was correlated to the literature and the structures determined.

## DISCUSSION

*Stereum* species like most basidiomycetes produce biologically active secondary metabolites when grown in laboratory defined submerged nutrient cultures (Lorenzen and Anke, 1998). Such compounds have been shown to have mosquito larvicidal activities from submerged cultures of the mushroom *Cyptotrama asprata* (Njogu et al., 2009). Here extra-cellular secondary metabolites from

cultures of *C. asprata* led to the purification of a strongly larvicidal compound; (oxiran-2-yl)methylpentanoate. This compound showed strong larvicidal activity against the *A. aegypti* larvae with $LC_{50}$ of 1.50 ppm and an $LC_{90}$ of 1.90 ppm after 24 h. The findings were found to mirror the results reported in this paper, where the extra-cellular secondary metabolites were in the culture filtrate of *Stereum* species (JO5289). Like in the case of *C. asprata*, the crude extracts from *Stereum* species (JO5289) were purified using column chromatography to give three compounds; tyrosol (1), 3-methoxy-5-methyl-1,2-benzenediol (2) and 2-hydroxy-4-(4-hydroxy-chroman-7yl) but-3-enal (3). These compounds were found to have larvicidal activity against *A. aegypti*.

From literature reports tyrosol (1) had been obtained from mycelia of an ascomycetes fungus *Cordyceps ophioglossoides* as an estrogenic substance (Kawagishi et al., 2004). It has also been obtained from phytopathogenic fungus *Ceratocystis adipose* as an antioxidant (Guzman-Lopez et al., 2007). Its production had been reported in other *Ceratocystis* species before, including *Ceratocystis fimbriatacoffea* (Gremand and Tabacchi, 1996), *Ceratocystis clarigera, Ceratocystis ips* and *Ceratocystis huntii* strains (Ayer et al., 1986).

Tyrosol has been reported to have pharmacological activity particularly antioxidant activity. It has also been used in atheroscelerosis treatment, protecting low density lipoproteins (LDL) from oxidation which play a role in the initiation and progress of cardiovascular diseases (Guzman-Lopez et al., 2007). Phytotoxic activity of tyrosol has been observed in lettuce leaves and certain toxicities in mice (Ayer et al., 1986). However, to our knowledge this was the first time the larvicidal activity was reported for tyrosol.

Compound 3 is a chromanone derivative and such compounds have been isolated from fungi and plants (Lee et al., 2007). Among the known naturally occurring chromanones, nearly all have alkyl substituent at the C2 or C3 position. They have also been synthesised by chemical methods for their extensive bio-activities such as antifungal, antibacterial, antitumour and antiviral activities (Li et al., 2007). The three compounds (1, 2 and 3) belong to two classes of compounds, the phenols and chromans (a derivative of flavonoids). The observed $LC_{50}$ and $LC_{90}$ values were significant and were within the range of those previously isolated larvicidal compounds reported from the literature. Ocimenone, a monoterpenoid isolated from *Tagetes minuta* oil exhibited $LC_{50}$ value of 40 ppm and a triterpene from *Azadiracta indica* showed an $LC_{50}$ value of 21.0 ppm (Geris et al., 2008). The observed $LC_{50}$ and $LC_{90}$ values for compounds 1, 2 and 3 were significant since they were within the range of those previously isolated larvicidal compounds reported from the literature. We wish to state that these com-pounds have no mammalian toxicities reported in the literature.

In conclusion the findings from this study reveal that cultures of *Stereum* species (JO5289) have larvicidal compounds that can be used to control mosquitoes and by extension control of spread of diseases by the vectors.

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*Full Length Research Paper*

# *Aphanomyces frigidophilus*, fungus-like organisms isolated from water of springs in Białystok, Poland

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**Investigations into occurrence of fungus *Aphanomyces frigidophilus* in water of springs Dojlidy Górne, Jaroszówka and Pietrasze within the town Białystok in Podlasie Province, Poland were conducted in Winter, Spring, Summer and Autumn of the year 2005. Samples were processed in the laboratory by routine methods commonly used to isolate these organisms. Bait method with the use of hemp seeds *Cannabis sativa,* small pieces of snake skin *Natrix natrix* and exuviae of shrimp *Gammarus* sp. as bait was applied to isolate the fungus *Aphanomyces frigidophilus* from the springs. The isolate was maintained on Potato Dextrose Agar PDA and stored in the culture collection of the Real Jardín Botánico CSIC Madrid, Spain. *Aphanomyces frigidophilus* occurred in 18 [(6)16.7%) in Winter, 3(8.3%) in Spring, 2(5.6%) in Summer, 7(19.4%) in Autumn, 2005] of the examined water samples. In Spring Dojlidy Górne it was very common and was found in all research seasons. The isolate was characterized by studding sequencing the internal transcribed spacer of nuclear DNA (ITS1+5.8S+ITS2). The results indicated the sequence comparisons of two ITS nuclear DNA for species identification: *Aphanomyces frigidophilus* 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence. The results indicated the sequence of our isolate corresponded to the species *Aphanomyces frigidophilus* (AY647192, version AY647192.1; GI: 48766837).**

**Key words:** *Aphanomyces frigidophilus,* snake skin of *Natrix natrix* and exuviae of shrimp *Gammarus* sp.

## INTRODUCTION

Water mould constitute a common group of organisms found in a variety of water ecosystems. Some of them are animal or human parasites. In favorable conditions, water mold acting as saprobionts can assume pathogenic properties, being a potential source of infection (Dick, 2001; Czeczuga et al., 2004a; b; Kiziewicz and Kurzatkowska, 2004; Kirk et al., 2008). In spring of rivers, a lot of representatives of water moulds of the class Oomycetes are met. The phylogenetic relationship of Oomycetes (watermolds) to fungi has been debated for many years. Oomycota (oomycota means egg fungus) or Oomycetes have been for a long time recognized as significantly different from the organisms classified as the Phylum Oomycetes in the Kingdom Fungi (True fungi) (Alexopoulos et al., 1996). Scientific studies have shown that some organisms may look like fungi yet are not really members of the Kingdom fungi. A cladistic classification based on modern insights supports a relatively close

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**Abbreviations: PDA,** Potato Dextrose Agar; **ITS,** internal transcribed spacer; **PCR,** polymerase chain reaction; **NCBI,** National Centre of Biotechnology Information.

relationship between Oomycetes with photosynthetic organisms such as brown algae and diatoms, within the heterokonts. The Oomycetes have been differently classified by numerous taxonomists, for instance they have been classified as heterokont organisms in the Kingdom Stramenopila, Phylum Heterokonta, Class Peronosporomycetes (Alexopoulos et al., 1996; Dick, 2001).

De Bary in 1860 (de Bary, 1860) established a new genus *Aphanomyces* to include a number of saprotrophic and parasitic plants observed during this period. The genus *Aphanomyces* comprising approximately 35-40 species is a smaller and less frequently encountered genera of the Saprolegniales order, Oomycetes Class (Scott, 1961; Uribeondo et al., 2009). A few species are parasites and responsible for economical important diseases affecting agriculture and aquaculture crops as well as wildlife populations of freshwater animals (Papavizas and Ayers, 1974; Söderhall and Cerenius, 1999).

The main task of the present study was to assess distribution of fungus-like organisms *Aphanomyces frigidophilus* from water of three limnocrenic springs situated in Białystok, Podlasie Province, Poland.

## MATERIALS AND METHODS

### Study area

Investigations into occurrence of fungus *A. frigidophilus* in water of springs Dojlidy Górne, Jaroszówka and Pietrasze within the town Białystok were conducted in Winter, Spring, Summer and Autumn of the year 2005. Spring Dojlidy Górne (53°06'N, 23°12'E) of Biała River, located in the eastern part of Białystok, Poland. Limnokrenic type, an artificial basin: area 0.380 km$^2$, max, width 0.65 m, depth 0.12 m, discharge 2.4 dm$^3$ s$^{-1}$, surroundings without trees. Spring Jaroszówka (53°10'N, 23°11'E) of Jaroszówka River, located in the north part of Białystok, Poland. Limnokrenic type, an artificial basin: area 0.340 km$^2$, max, width 0.60 m, depth 0.12 m, discharge 2.4 dm$^3$ s$^{-1}$, surroundings without herbaceous and trees. The spring is surrounded by cultivated fields. The bed is covered with sand. Spring Pietrasze (53°10'N, 23°9'E) of River Biała, located in the north part of Białystok, Poland, limnokrenic type, an artificial basin: area 0.290 km$^2$, max, width 0.60 m, depth 0.12 m, discharge 2.5 dm$^3$ s$^{-1}$, surroundings without herbaceous and trees.

### Microbial analyses

Microbial analyses was made in the laboratory, The Real Jardín Botánico CSIC, Madrid, Spain of the year 2006. For the microbial analysis of fungi samples of water were collected from each sampling site of research springs of rivers. The water collected from the respective reservoirs was poured in sterile conditions into plastic bottles, and placed in the laboratory. All containers were incubated at 13 ±2°C, with access to daylight resembling natural conditions and following the recommended instructions (Seymour and Fuller, 1987). Water limnokrenic type of springs in Poland have almost constant temperature throughout the year. The water temperature the research springs was about 9°C throughout the period of the study. pH of springs of water was neutral close to the acid.

Samples were processed in the laboratory by routine methods commonly used to isolate these organisms. Bait method with the use hemp seeds *Cannabis sativa* L., small pieces of snake skin *Natrix natrix* and exuviae of shrimp *Gammarus* sp. as bait was applied to isolate the fungus *A. frigidophilus* from the springs. Water samples (100 ml) of each site were homogenized and four aliquots of 25 ml were placed in Petri dishes of 9 cm diameter with sterile baits. Dishes were stored in the laboratory at room temperature 13 ±2°C for 4-5 days (Seymour and Fuller, 1987). The colonized fragments of hemp-seeds, shrimp and snake skin were transferred to new Petri dishes which contained sterilized, filtered spring or distilled water and crystalline penicillin (2000 units per litre of water) to inhibit bacterial growth. Dishes were microscopically examined weekly for up to three weeks in order to identify the genus or species. The isolate was maintained on Potato Dextrose Agar (PDA) (Merck Cat. No. 1.10130.0500, Merck KCaA 64271 Damstadt, Germany) (Unestam, 1965) and stored in the culture collection of the Real Jardín Botánico CSIC, Madrid, Spain. Morphological characters of asexual structures and measurements were made microscopically on material mounted in water. Fungi were successively observed under an optic microscope Olympus BX 51 (100, 400 and 1000x magnification). The respective stages of the fungal development were evaluated by means of an ocular micrometer. Several microscopic preparations were made from each sample.

### Isolates and internal transcribed spacer (ITS) sequences

Sequences were obtained from pure cultures, ITS-polymerase chain reaction (PCR)-based specific tests from GenBank representing a total of 12 *Aphanomyces* spp. All the isolates were growing on PDA (Merck Cat. No. 1.10130.0500, Merck KCaA 64271 Damstadt, Germany) (Unestam, 1965) for 3-5 days at room temperature as described by Cerenius et al., (1987). Mycellia pellets were washed with sterile water collected in 1.5 ml microcentrifuge tubes and stored at 20°C before DNA extractions. The origin of the isolates and their reference numbers were coded with the initials SAP and are maintained in the Oomycetes culture collection of the Real Jardín Botánico CSIC, Madrid. In this study, was used the following sequences as reference for species *A. frigidophilus* Kitanch, and Hatai, AY647192.

### DNA extraction and PCR amplification

For DNA extraction, mycelium was grown as drop cultures (Cerenius and Söderhall, 1985) and from them, genomic DNA was extracted using an Easy Nucleic Acid® (EZNA) (Fungal DNA Miniprep Kit (Omega Biotek, Doraville, USA) as described in the study of Martín and García-Figueres (1999). DNA fragments containing internal transcribed spacers ITS1 and ITS2 including 5.8S gene of the nuclear DNA was amplified with primer pairs ITS5/ITS4 (White et al., 1990) primers as described in Martin et al., (2004). Nucleotide BLASTN searches with option Standard nucleotide BLAST and BLASTN 2.6 were used to compare the sequence obtained against the sequences in the National Centre of Biotechnology Information (NCBI) nucleotide databases.

### Hyphal growth rates and repeated zoospore emergence

Selected isolates of parasitic and saprotrophic/opportunistic species were inoculated in Potato Dextrose Agar PDA (Unestam, 1965) and incubated at 20°C. Colony diameter was measured every 24 h during maximum period of 3 days. Briefly, mycelia were grown in PDA drop cultures for 3 days at 20°C. To trigger sporulation, mycelia were washed thee times with sterile water and incubated in petri dishes containing sterile water for 14 at 15°C to allow zoospore release. The release of new zoospores was observed under the microscope after incubating the cyst suspension at 15°C for 150 min.

**Table 1.** Distribution and seasonal occurrence of aquatic fungus-like organisms *Aphanomyces frigidophilus* in 36 samples from three different sites of springs in Białystok (n =3).

| Water reservoirs | Number of collected water samples | Seasonal occurrence/number of water samples where fungus was found | | | | |
|---|---|---|---|---|---|---|
| | | Winter | Spring | Summer | Autumn | Together |
| Dojlidy Górne | 12 | 3 | 2 | 1 | 3 | 9 |
| Jaroszówka | 12 | 2 | 1 | | 2 | 5 |
| Pietrasze | 12 | 1 | | 1 | 2 | 4 |
| Total number of samples | 36 | 6 | 3 | 2 | 7 | 18 |
| Percentage % | 100 | 16.7 | 8.3 | 5.6 | 19.4 | 50.0 |

#### Production of sexual structures

*Aphanomyces* isolates were cultivated on sterile substrates including hemp seeds (*C. sativa* L.), exuviae of *Gammarus* sp., and snake skin *N. natrix* in petri dishes containing autoclaves, sterilized water (one bait per dish) and incubated in the dark at 20°C. Dishes were examined periodically (for 3 weeks) for evidence of growth with a stereo microscope and to verify the presence or absence of sexual structures. All isolates were characterized and identified according to the study of Scott (1961). Taxonomic identifications were made according to the study of Scott (1961), Batko (1975), Pystina (1994) and Seymour and Fuller (1987).

## RESULTS

The study conducted in springs located in Białystok, Poland showed the occurrence of fungus-like organisms *A. frigidophilus* Kitanch and Hatai in samples of water reservoirs. As shown in Table 1, *Ap. frigidophilus* occurred in all three springs of examined waters. It occurred in 18 [(6)16.7%) in Winter, 3(8.3%) in Spring, 2(5.6%) in Summer, 7(19.4%) in Autumn, 2005] of the examined 36 water samples. Compared to Spring Jaroszówka and Pietrasze in Spring Dojlidy Górne it was very common and was found in all research seasons. The results indicated the sequence comparisons of two ITS nuclear DNA for species identification: *Ap. frigidophilus* 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8 S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence. The results indicated the sequence of our isolate corresponded to the species *Ap. frigidophilus* (AY647192, version AY647192.1; GI: 48766837).

## DISCUSSION

An optical microscope was used for microscopic observations with total magnifications of 100, 400 and 1000 times. Scientific classification: *Ap. frigidophilus* Kitanch. and Hatai 1997 belonging to the Domain Eukarya, Supergroup/Subkingdom Chromalveolata, Kingdom Chromista=Stramenopila, Phylum Heterokontophyta, Class Oomycota, Order Saprolegniales, Family Saprolegniaceae, Genus *Aphanomyces* (Kirk et al., 2008). Genus *Aphanomyces* is water and soil saprotrophs of plants and animals. Facultative parasites of algs, aquatic zoosporic fungi from genus *Achlya*. There are around 35-40 described *Aphanomyces* species which occur in very different ecological niches ranging from specialized plant or animal parasites with a narrow host range, to saprotrophic species growing on decaying animal and plant debris (Scott, 1961; Dick, 2001; Johnson et al., 2002; Uribeondo et al., 2009). Hyphae are usually thin, highly branched, 3-8 µm in diameter, sometimes ragouts (*Ap. frigidophilus*). Zoosporangia morphologically identical in each species. Zoospores 7-9 µm in diameter. Oogonia predominantly single, 18-33 µm in diameter, with delicate (*Aphanomyces laevis*), wrinkled (*Aphanomyces irregularis*) or granular (*Ap. frigidophilus*) wall, spherical with conical spine-like projection, placed on short, lateral hyphae (*Aphanomyces stellatus*). Cytospores 7-10 µm in diameter (*Ap. laevis*). Oospores subcentric (*Ap. frigidophilus*), with thick wall (*Ap. laevis*), 15-29 µm in diameter, predominantly single or 2 in number, filling the oogonium. Antheridia - one (*Ap. stellatus*) or more around one oogonium (*Ap. laevis*, *Ap. stellatus*), diclinous or monoclinous, rarely androgynous (*Ap. laevis*, *Ap. stellatus*) (Johnson et al., 2002).

*Ap. frigidophilus* has the hyphae with rounded hyphal tips, hyphe thin in 5-7 µm in diameter, aseptate, sporangia long with a single row. Primary zoospores near the orifice emerged in this manner, whereas those emerging later became elongate with rounded ends during the passage through zoosporangium. Secondary zoospores were reniform, laterally biflagellate. Oogonia were abundant on short oogonial stalk, 16-25 µm diameter. Young oogonia with rough-ended outer contours, elongated oogonia between hyphae. Mature oogonium showing single subcentric oospore 14-22 µm diameter, with a large shiny vesicle surrounded by fine granules, the outer contours have short papillate or irregular (Kitancharoen and Hatai, 1997).

However, fungi in the genus *Aphanomyces* have been

occasionally reported on fish eggs. *Ap. laevis* is only species of this genus that has been reported on eggs of rainbow trout *Onchorhynchus mykiss* (Scott and O' Bier, 1962) and vendance *Coregonus albula* (Czeczuga and Woronowicz, 1993), although an unidentified *Aphanomyces* was also reported on rainbow trout (Scott and O'Bier, 1962). The first *A. frigidophilus* has been reported only from Japan. *Ap. frigidophilus* was detected on the eggs of Japanese char *Salvelinus leucomaenis* from Tochigi Prefectural Fisheries Experimental Station, Utsonomiya, Japan (Kitancharoen and Hatai, 1997, 1998). The first in Poland and in Europe *Ap. frigidophilus* have been described only on the basic morphological studies by Czeczuga et al., (2004a; b) in fish eggs of European Whitefish *Coregonus lavaretus holsatus* obtained from Lake Wdzydze in Kaszuby (Poland). The next in Europe *Ap. frigidophilus* was described on crayfish cuticule *Austropotamobius pallipers* by Ballesteros et al. (2006), too. Dead crayfish were collected in river Tajuňa, Guadalajara (Spain). The fungus was grown on fish eggs of European Whitefish in water of Cypiesk Spring and Akcent Pond in Białystok, Poland. Worth a special note was the finding *Ap. frigidophilus*, new to Polish freshwaters (Czeczuga et al., 2004a; b). The fungus was growing also on the eggs of sea trout *Salmo trutta* m. trutta in running water from River Biała, Krasna and Supraśl near Białystok (Poland) (Czeczuga et al., 2005). *Ap. frigidophilus* was the most common straminipilous organisms found on the eggs of Atlantic salmon *Salmo salar.* The investigated eggs were collected from 60 females of Atlantic salmon caught during their spawning migration in Darłowo town on the River Wieprza (wild form), and Świbno town on the River Vistula (wild form), and from those bred in fresh water in hatcheries at Miastko town (farmed form). *Ap. frigidophilus* was found growing on the eggs of Atlantic salmon from 14 females from research rivers such as Wieprza and Vistula and from hatcheries in Miastko (Czeczuga et al., 2011).

Molecular phylogenetic relationships among 12 species of *Aphanomyces* de Bary were analyzed based on 108 ITS sequence s of nuclear DNA by Uribeondo et al., (2009). Within *Aphanomyces* clade, three main lineages were found: plant parasitic, animal parasitic and saprotrophic or oportunic parasitic. The animal parasitic lineage had low support and comprised sequences from *Aphanomyces* spp. that are parasites of animals, or have been isolated from animals, and isolates of the saprotrophic species *Ap. stellatus.* This lineage contained the reference sequences for *Aphanomyces astaci, Ap. frygidophilus, Ap. invadans, Ap. piscicida* and *Ap. stellatus.* This lineage comprised species *Aphanomyces* that thrive in freshwater or estuarine aquatic environments, and included all species parasitic to animals, the animal associated species *Ap. frigidophilus*, and saprotrophic species *Ap. stellatus.* The *Ap. frigodophilus* clade comprised the sequences of reference for *A. frigidophilus* (AY647192). Isolates originated from Japan and Spain with a wide host range that included salmonids (Japan) and freshwater crayfish (Spain) (Kitanchaoren and Hatai, 1997; Ballesteros et al., 2006; Uribeondo et al., 2009). Although, isolates of this species have always been obtained from diseased aquatic animals, it is uncertain whether this species is a parasitic or opportunistic (Uribeondo et al., 2009).

The results indicated the sequence comparisons of two ITS nuclear DNA for species identification. The results indicated the sequence of our isolate corresponded to the species *Ap. frigidophilus* (AY647192, version AY647192.1; GI: 48766837). GenBank sequence AY647192.1 corresponding to strain NJM9500 of *Ap. frigidophilus* directly submitted by Phadee et al. (2004). Thus, this study represents the first isolation of *Ap. frigidophilus* in fresh waters in Poland. This parasitic fungus considered among one hundred worst invasive species (Global Invasive Species Database, 2005) and is responsible for the decline of the indigenous European freshwater animals not only to crayfish, and also fish which are currently endangered in Europe and specially in Poland (Czeczuga et al., 2004a; b, 2005; Ballesteros et al., 2006; Uribeondo et al., 2009).

## Conclusion

The study conducted in springs of rivers located in Białystok, Poland showed the occurrence of fungus-like organisms *Aphanomyces frigidophilus* Kitanch. and Hatai in samples of water reservoirs. The results indicated the sequence comparisons of two ITS nuclear DNA for species identification. The results indicated the sequence of our isolate corresponded to the species *Ap. frigidophilus*. Thus, this study represents the first isolation (on the basic molecular studies) of *Ap. frigidophilus* in fresh waters in Poland.

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*Full Length Research Paper*

# Activity guided isolation and characterization of antioxidant and antibacterial agents from some local Nigerian plants

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**This study aimed to present the activity guided fractionation, isolation and characterization of antioxidants and antibacterial agents from combined mixture of plants (*Vitex doniana*, *Diospyros mesipiliformis*, *Acacia polycantha*, *Pirinari macrophylla*, *Ficus sycomorus* and *Parkia biglobosa*) and that of *Pergularia tomentosa.* Combined Mixture of Plants (CMP) is used locally in ratio of 1:1 for the treatment of bacterial infections. The CMP and *P. tomentosa* were extracted with methanol separately; the residues obtained were also separately suspended in water and successively fractionated with hexane, ethylacetate and n-butanol. All the fractions obtained were screened for antimicrobial and antioxidant activities. For CMP, only the ethyl acetate fraction (EF) indicated marginal antibacterial activity with 8.0, 7.0 and 7.0 mm zone of inhibition against *Micrococcus luteus* (MTCC 2470), *Bacillus subtilis* (MTCC 121) and *Salmonella typhimurium,* respectively. Minimum inhibitory concentration (MIC) for the CMP was greater than 1000 for *M. luteus* and *S. typhimurium* and 87.5 µg/ml for *B. subtilis.* The CMP fraction was subjected to chromatographic separations which resulted in the isolation and characterization of five bioactive constituents, gallic acid, 3β-OH-α-amyrin, 5,7,3'.4',5'-pentahydroxy-3-O-glucophyranoside flavones (myricetin 3-O-β-rhamnopyranoside), 5,7,3',4' tetrahydroxy-3-O-glucopyranoside flavone (quercetin 3-O-β-rhamnopyranoside) and 3,5,7,3',4'-pentahydroxy flavones (quercetin). They were characterized with the help of ESI-MS, IR, $^{1}H$ $C^{13}$, HMBC/HSQC and COSY-NMR data. These compounds did not show antibacterial activity when tested separately but exhibited appreciable antioxidant activities in different manner. Chromatographic fractionation of hexane extract of *P. tomentosa* resulted in the isolation of lupeol acetate (LA) with marginal but selective activity against *M. luteus* and the activity is due to LA rather than the combined constituents. These findings suggest that the fractions of the extracts and pure compounds possess antibacterial and antioxidant properties.**

**Key words:** Antioxidant properties, antibacterial activity, NMR data, *Pergularia tomentosa*, combined mixture of plants.

## INTRODUCTION

Diseases caused by pathogenic bacteria and fungi present critical problem to human health and are one of the main causes of morbidity and mortality worldwide (WHO, 1998). Resistance to antibiotics and the occur-

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rence of toxicity during prolonged treatment with present day drugs have been the reasons for extended search for newer drugs to treat microbial infections (Fostel and Lartey, 2000). Drug resistance is on the increase and there is need to search for other antimicrobial agents (Sharma and Kumar, 2006; Negi and Dave, 2010). Combination therapy is an alternative approach in the search for novel compounds with ability to deal with antibiotic resistant microorganisms. The combination can be of different plant extracts or plant extracts with standard antibiotics or chemicals. Studies have shown that plant extracts in combination of two or more are yielding effective antimicrobial activity against several microorganisms that even include drug resistant bacteria (Karmegam et al., 2008). Thus, interviews with traditional healers in Sokoto, Nigeria, indicated the use of the six plants in combination of 1:1 in the treatment of bacterial infections without any scientific validations. Plants have been used to treat infectious diseases due to their antimicrobial properties. This is due to the presence of various kinds of phytochemicals including phenolic compounds, alkaloids, terpenoids and essential oils (Lewis and Elvin-Lewis, 1995; Cowan, 1999).

*Pergularia tomentosa* (PS milk weed) is used in Northern Nigeria for tanning and treatment of skin diseases. Its isolated cardenolides have been shown to cause apoptotic cell death of Kaposi's sarcoma cells (Hamed et al., 2006). The roots have found applications in the treatment of bronchitis, constipation and skin diseases (Hammiche and Maize, 2006). It is well known that free radicals cause cell damage through mechanism of covalent binding and lipid peroxidation with subsequent tissue injury (Osawa et al., 1990). Antioxidant agents of natural origin have attracted special interest because they can protect human body from free radicals. Antioxidant properties of certain flavonoids of plant origin have already been established (Di Carlo et al., 1999). *Ficus sycomorus* is used locally for antimicrobial treatment in Nigeria and has been reported to have antimicrobial activities (Hassan et al., 2007). In the present work, we evaluated the synergistic antibacterial properties of the combined mixture of plants (CMP) and isolated and characterized the bioactive principles of the CMP and *P. tomentosa*. The pure compounds were also screened for antibacterial and antioxidant properties. To the best of our knowledge these have not been reported so far. Therefore, it is worthwhile in this study to present the activity guided fractionation, isolation and characterization of antioxidants and antibacterial agents from the CMP and *P. tomentosa*.

## MATERIALS AND METHODS

### Plant material

The leaves, roots and stems of the selected plants were collected from the adjoining area of Usmanu Danfodiyo University (UDU), Sokoto, Nigeria. After proper taxonomic identification of all the plants (before combination) by the Taxonomist of Botany Unit (U.D.U.), the plant parts (leaf, root and stem) were open air-dried under the shade and pulverized into a moderately coarse powder.

### Chemicals

DPPH, ascorbic acid, quercetin and $FeCl_3$ were purchased from Sigma Chemical Co. (St. Lois, MO, USA). Vanillin from BDH, Follin Ciocalteu‘s phenol reagent and sodium carbonate were from Merck Chemical supplies (Darmstadt, Germany). All the chemicals and solvents used were of analytical grade.

### Microbial organisms

The microbial organisms used were available in the Molecular and Bio-prospection Unit, of Central Institute of Medicinal and Aromatic Plants, Lucknow, India. The bacterial isolates were maintained on nutrient agar medium.

### General experimental procedures

The $^1H$ and $^{13}C$ NMR spectra were recorded on a Bruker Avance 300 (300 MHz). Column chromatography was performed with silica gel (60 to 120 mesh). TLCs were run on ready-made aluminum sheets (silica gel 60 F254, 0.25 mm, 20 $\times$ 20 cm, Merck, Germany) while preparative TLCs were run on glass plates (silica gel 60 F254, 0.5 mm, glass plates 20 $\times$ 20 cm) from Merck, Germany. Spots on the TLC plates were visualized by spraying with vanillin sulfuric acid and heating the plate in oven for 5 min at 100°C. Vacuum liquid chromatographic (VLC) separation was run over silica gel H (average particle size approximately 10 µm). The powdered leaf of *P. tomentosa* (PT) and the parts of the combined mixture of plants (1:1) were extracted with methanol separately and each residues obtained were dissolved in water separately and each were further fractionated with hexane, petroleum ether and n-butanol. The following fractions were obtained:

*P. tomentosa* (methanolic leaf) extract = 28.9 g → HF (g), EF (g), BF (g),
Combined mixture of plants = 40.0g → HF (g), EF (g), BF (g).

### Isolation of bioactive compounds from the ethyl acetate fraction (EF) of combined mixture of plants (CMP)

The ethyl acetate fraction (EF) of combined mixture of plants that showed remarkable antibacterial and antioxidant activities was further fractionated. Eight grams (8 g) of this fraction was subjected to vacuum liquid chromatographic (VLC) separation over silica gel H (average particle size approximately 10 µm). Stepwise gradient elution was carried out with hexane, hexane-chloroform, chloroform, chloroform-methanol and methanol. A total of 249 fractions were collected. The fractions were pooled on the basis of their TLC profile as follows: Fractions 14 to 62 (270 mg), fractions 63 to 73 (308 mg), fractions 152 to 184 (325 mg), fractions 201 to 214 (524 mg), fractions 215 to 230 (495 mg), fractions 231 to 246 (1000 mg).

### Isolation of bioactive compounds from the hexane fraction of *Pergularia tomentosa*

Separately, activity guided separation of hexane fraction of *P. tomentosa* which showed antibacterial activity was carried out. After series of chromatographic separation, a total of 114 ddfractions were collected. The fractions were pooled on the basis of their TLC

profiles and the hexane fraction resulted in the isolation of lupeol acetate (Figure 6) and its antibacterial activity was determined.

**Antioxidant activity**

***Free radical scavenging activity***

It was measured using the modified method of Blois (1985). DPPH (50 µL of 0.1 mM dissolved in methanol) was added to the tested compounds at different concentrations (1, 5, 10, 25, 50 and 100 µg) and 40 µL of Tris-HCl were also added. Equal volume of methanol, Tris-HCl and DPPH were added in the control test. The mixture was shaken vigorously and incubated at 37°C for 20 min. The absorbance at 517 nm was measured spectrophotometrically. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percentage of scavenging of DPPH was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{AO - A1}{AO} \times 100$$

Where, AO is the absorbance of the control reaction, A1 is the absorbance in the presence of the sample.

***Total phenolics estimation***

The amount of total phenolics was determined by Folin-Ciocalteu's colorimetric method (Wolf et al., 2003). Briefly, the concentration of the compounds (1, 5, 10, 25, 50 and 100 µg) were mixed with 50 µL of distilled water and 250 µL of Folin-Ciocalteu's reagent were added and mixed properly. A 250 µL of sodium carbonate was then added. The mixture was incubated at 37°C for 90 min and the absorbance was measured at 765 nm by a XPLORER XP2001 spectrophotometer. Gallic acid was used as a standard and total phenolics were expressed as grams of Gallic acid equivalent (g of GAE) per 100 g of fresh weight.

***Nitric oxide scavenging activity***

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess reaction (Green et al., 1982; Marcocci et al., 1994). The reaction mixture containing 100 µL of sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and the compounds (1, 5, 10, 25, 50 and 100 µg) were incubated at room temperature for 30 min. After incubation, 50 µL of incubated reaction mixture were added to 100 µL of Griess reagent (1:1 sulfanilamide: naphthylethylene diaminehydrochloride). The absorbance of the chromophore formed was measured at 546 nm. The percentage of nitric oxide scavenging activity was calculated using the following equation:

$$\text{\% inhibition} = \frac{AO - A1}{AO} \times 100$$

Where, AO is the absorbance of the control reaction, A1 is the absorbance in the presence of the sample.

***Total antioxidant capacity***

The assay was based on the reduction of molybdenum (VI) to molybdenum (V) by the compounds and the subsequent formation of a green phosphate Mo (V) complex to acid pH (Priesto et al., 1999). Compounds (1, 5, 10, 25, 50 and 100 µg) were combined with 1 ml of total antioxidant capacity (TAC) reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95°C for 90 min and cooled down to room temperature. The absorbance was measured at 695 nm against reagent blank. The total antioxidant capacity was expressed as the number of equivalent of ascorbic acid (mg/g of dry mass).

***Reducing power***

The reducing power of the extract/compound was determined according to the method of Oyaizu (1986). Different concentrations of the compounds (1, 5, 10, 25, 50 and 100 µg) were mixed with 250 µL phosphate buffer (pH 6.6, 0.2 M) and 250 µL (1%) potassium ferricyanide. The mixture was incubated at 50°C for 20 min. A 250 µL of trichloroacetic acid (10%) was added to the mixture and centrifuged at 5000 rpm for 3 min. Then 250 µL of the supernatant was mixed with 250 µL of distilled water and 50 µL of $FeCl_3$ (0.1%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated reducing power.

***Total flavonoids***

Estimation of total flavonoids was done according to the method of Ordon Ez et al. (2006). To 50 µL of the compounds (1, 5, 10, 25, 50 and 100 µg), 150 µL of methanol, 10 µL of $AlCl_3$, 10 µL of potassium acetate and 280 µL of distilled water were added. The mixture was incubated at room temperature (25 to 37°C) for 30 min. The absorbance of the reaction mixture was measured at 415 nm. A yellow color indicated the presence of flavonoids content. Total flavonoids content was calculated as quercetin (mg/g).

***FRAP assay***

The stock solution included 300 mM acetate buffer (3.1 g $C_2H_3$ $NaO_2.3H_2O$ and 16 ml $C_2H_4O_2$) pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-5-triazine) solution in 40 mM HCl and 20 mM $FeCl_3.6H_2O$. The fresh working solution was prepared by mixing 2.5 ml acetate buffer, 2.5 ml TPTZ and 2.5 ml $FeCl_3$ (FRAP reagent). A 50 µL of the compound was added to 1.5 ml of FRAP reagent. The mixture was mixed and incubated at 37°C for 5 min. Absorbance was measured at 593 nm. Results were expressed as $FeSO_4$ equivalent (Benzie and Strain, 1996).

***Disc diffusion assay***

The CMP and *P. tomentosa* extracts and pure compounds were screened for antibacterial activity against the following organisms: *Staphylococcus aureus* (MTTC 96), *Staphylococcus aureus* (MTTC 2940), *Escherichia coli* (MTTC 739), *Micrococcus luteus* (MTCC 2470), *Bacillus subtilis* (MTCC 121), *Streptococcus mutants* (MTCC 890), *Raoultella planticola* (MTCC 530), *Klebsiella pneumoniae* and *Salmonella typhimurium*. Strains were grown overnight at 36°C in nutrient broth medium. Inoculums for the assays were prepared by diluting cell mass in 0.85% NaCl solution, adjusted to McFarland scale 0.5 ($1.5 \times 10^8$ CFU/ml) and prepared nutrient agar plates were seeded with $1.5 \times 10^8$ CFU/ml suspensions of test bacteria. The antibacterial activity of culture was determined using disc diffusion assay according to the Clinical and Laboratory Standard Institute (CLSI, formerly NCCLS). Absorbent disc (5 mm) were impregnated with 5 µL of the CMP and *P. tomentosa* extracts (100

**Table 1.** Antibacterial activity of ethyl acetate fraction of combined mixture of plants and the isolated compounds.

| Parameter | SA-96 | SA-2940 | ML | EC | BS | SM | STM | KP | RP |
|---|---|---|---|---|---|---|---|---|---|
| Plant extract/pure compounds/drugs | | | | | | | | | |
| CMP | - | - | 8.0 | | 7.0 | - | 7.0 | - | - |
| 73-91LPE | - | - | 10.0 | | - | - | - | - | - |
| 45-46 cmp | - | - | - | - | - | - | - | - | - |
| 126-141 | - | - | - | - | - | - | - | - | - |
| 128-157 cmp | - | - | - | - | - | - | - | - | - |
| 79-83 | - | - | - | - | - | - | - | - | - |
| cp 87-112 | - | - | - | - | - | - | - | - | - |
| Kanamycin | 22 | 20 | 24 | 23 | 35 | 17 | 29 | 5 | 25 |
| Ampicillin | 23 | 24 | 25 | 5 | 32 | 5 | 25 | 5 | 5 |

Zone of inhibition are recorded in mm, - = no activity. SA = *Staphylococcus aureus* (MTTC 96 and MTTC 2940) and (MTTC 2940), EC = *Escherichia coli* (MTTC 739), ML= *Micrococcus luteus* (MTCC 2470), BS = *Bacillus subtilis* (MTCC 121), SM = *Streptococcus mutants* (MTCC 890), RP= *Raoultella planticola* (MTCC 530), KP = *Klebsiella pneumoniae* and STM = *Salmonella typhimurium.* CMP = combined mixture of plants (ethylacetate fractions), 73-91LPE (Figure 6), 45-46 cmp (Figure 2). 126-141 (Figure 4), 128-157 cmp (Figure 3), 79-83 (Figure 1), CP87-112 (Figure 5).

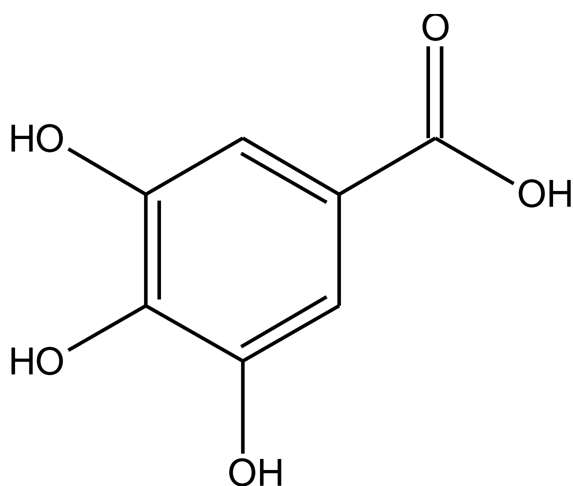


**Figure 1.** Fractions 79-83 = Gallic acid.

mg/ml) and pure compounds (10 mg/ml) and placed onto the surface of inoculated agar plates. Plates were incubated at 37°C for 24 h. Positive control discs of kanamycin and ampicillin was included. Antibacterial activity was expressed as the diameter of the inhibition zone (mm) produced by the extracts (Mellou et al., 2005).

### *Minimum inhibitory concentration*

The CMP and *P. tomentosa* extracts that showed some activity were subjected to MIC test. MIC test was carried out according to the method of Ellof (1998), using Muller-Hinton Broth on a tissue culture test plate (96 wells). The stock solutions of extracts were transferred into the first well, and serial dilutions were performed in order to have concentrations in the range of 1000 to 7.81 µg/ml. Inoculums for the assays were prepared by diluting cell mass in 0.85% NaCl solution, adjusted to McFarland scale 0.5, added to all wells and incubated at 36°C for 24 h. MIC was defined as the lowest concentration of the extracts that inhibited visible growth.

## RESULTS AND DISCUSSION

The results of antibacterial activity are presented in Table 1, which shows that only ethyl acetate fraction (EF) of combined mixture of plants (CMP) has marginal antibacterial activity with 8.0, 7.0 and 7.0 mm zone of inhibitions for *M. luteus*, *B. subtilis* and *S. typhimurium*, respectively. Minimum inhibitory concentration (MIC) for the CMP was greater than 1000 for *M. luteus* and *S. typhimurium* and 87.5 µg/ml for *B. subtilis.* Figures 1 to 5 did not show antibacterial activity. Lupeol acetate (Figure 6) was found to inhibit *M. luteus* (MTCC 2470) with inhibition zone of 10.0 mm. Lupeol acetate belongs to lupane type triterpenes and was reported to have antimicrobial activity (Prachayasittikul et al., 2010).

Our findings are consistent with the study on synergistic activity of six plants that showed activity against pathogenic bacteria by Karmegam et al. (2008). All the pure compounds isolated from the CMP extract did not show antibacterial activity. The antibacterial activity of the ethylacetate fraction of CMP was due to combination of all the constituents in the CMP rather than the individual compounds isolated. A series of chromatographic separation of ethylacetate fraction of CMP (as outlined in the experimental section) resulted in the isolation and characterization of bioactive constituents, Gallic acid (Figure 1), 3β-Hydroxy-α-amyrin (Figure 2), 5,7,3'.4',5'-pentahydroxy-3-O-glucophyranoside flavones (Figure 3), 5,7,3',4' tetrahydroxy-3-O-glucopyranoside flavones (Figure 4), 3,5,7,3',4'-pentahydroxy flavone (Figure 5). All the compounds isolated were characterized with the help of ESI-MS, IR, $^{1}H$ $C^{13}$, HMBC/HSQC and COSYNMR. Chemical analysis has indicated that some complex compounds elaborated by natural organisms may hardly be synthesized by chemical processes (Azas et al., 2002). However, the bacterial resistance to chemical

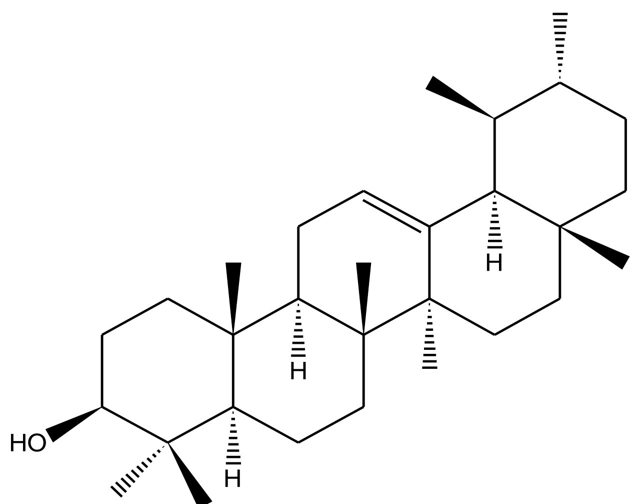


**Figure 2.** Fractions 45-46 cmp = 3β-Hydroxy-α-amyrin.

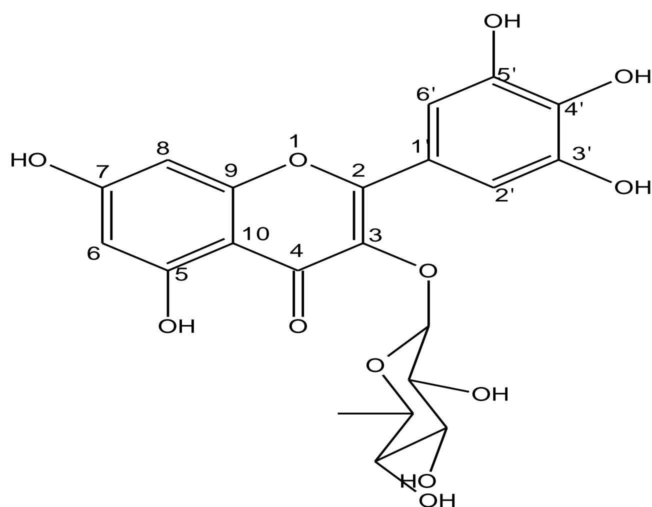


**Figure 3.** Fractions 128-157 cmp = 5,7,3'.4',5'-pentahydroxy-3-O-glucophyranoside flavones (myricetin -3-O-β-rhamnopyranoside).

treatment still remained important. Natural products isolated from the plants in the present study may be potential sources of new antioxidant drugs.

Activity guided separation of hexane fraction of *P. tomentosa* was also carried out. After series of chromatographic separation, a total of 114 fractions were collected. The fractions were pooled on the basis of their TLC profile and the hexane fraction resulting in the isolation of bioactive constituent (Figure 6) lupeol acetate (LA). LA was screened for its antibacterial activity, which showed marginal but selective activity against *M. luteus* (Table 1). This confirms that the lack of antibacterial activity of hexane extract of *P. tomentosa* was due to combination of all the constituents rather than LA alone.

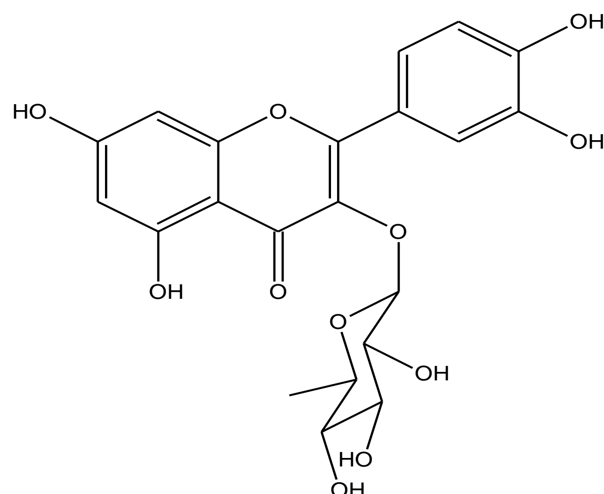


**Figure 4.** Fractions 126-141 = 5,7,3',4' tetrahydroxy-3-O-glucopyranoside flavone (quercetin- 3-O-β-rhamnopyranoside).

$^{1}$H NMR (CDCl$_3$, 300 MHz): δ 7.05 (2H, s, H-3 & H-7), $^{13}$C NMR (CD$_3$OD, 300 MHz): 170.42 (C-1'), 121.93 (C-1), 110.30 (CH, C-2), 146.35 (C-3), 139.57 (C-4), 146.35 (C-5), 110.30 (CH, C-6)

$^{1}$H NMR δ 0.77 to 1.14 (24H, 6x ter-Me & 2x sec-Me) 1.3 (1H, d, J = 6.3Hz, 18α-H), 3.20 (1H, dd, J = 5.1, 1.02 3α-H) 5.20 (1H, t, J = 3.0, 12-H)

$^{13}$C NMR (CDCl$_3$, 300 MHz): 38.76 (CH$_2$, C-1), 27.25 (CH$_2$, C-2), 78.98 (CH, C-3), 38.76 (C-4), 55.16 (CH, C-5), 18.34 (CH$_2$, C-6), 32.91 (CH$_2$, C-7), 39.99 (CH, C-8), 47.69 (CH, C-9), 36.88 (C-10), 23.25 (CH$_2$, C.11), 124.39 (CH, C-12), 139.30 (C-13), 42.03 (C-14), 28.08 (CH$_2$, C-15), 26.60 (CH$_2$, C-16), 33.73 (C-17), 59.04 (CH, C-18), 39.59 (CH, C-19), 39.59 (CH, C-20), 31.23 (CH$_2$, C-21), 41.51 (CH$_2$, C-22), 28.08 (CH$_3$, C-23), 15.62 (CH$_3$, C-24), 15.62 (CH$_3$, C-25), 16.84 (CH$_3$, C-26), 23.25 (CH$_3$, C-27), 28.08 (CH$_3$, C-28), 17.46 (CH$_3$, C-29), 21.39 (CH$_3$, C-30).

$^{1}$H NMR (MeOD, 300 MHz): δ 6.13 (1H, d, J = 2.0 Hz, H-6), 6.29 (1H, d, J = 2.0 Hz, H-8'), 6.89 (2H, s, H-2' & H-6'), 5.25 (1H, d, J = 1.5 Hz, H-1''), 3.43 (1H, dd, J = 5.7, 9.0 Hz, H-4''), 3.73 (2H, m, H-2'' & H-3''), 4.17 (1H, m, H-5''), 0.89 (3H, d, J = 6.0 Hz, H-6'').

$^{13}$C NMR (MeOD, 300 MHz): δ158.41 (C-2), 136.24 (C-3), 179.57 (C-4), 163.05 (C-5), 99.81 (C-6), 165.83 (C-7), 94.72 (C-8), 159.37 (C-9), 105.79 (C-10), 121.85 (C-1'), 109.59 (C-2'), 146.43 (C-3'), 137.83 (C-4'), 146.75 (C-5'), 109.59 (C-6'), 103.56 (C-2''), 71.80 (C-3'') 73.27 (C-4'') 79.45 (C-5'') 72.00 (C-6''), 17.62 (C-7'').

$^{1}$H NMR (MeOD, 300 MHz): δ 6.19 (1H, d, J = 2.2 Hz, H-6), 6.38 (1H, d, J = 2.2 Hz, H-8), 6.88 (1H, d, J = 8.4 Hz,

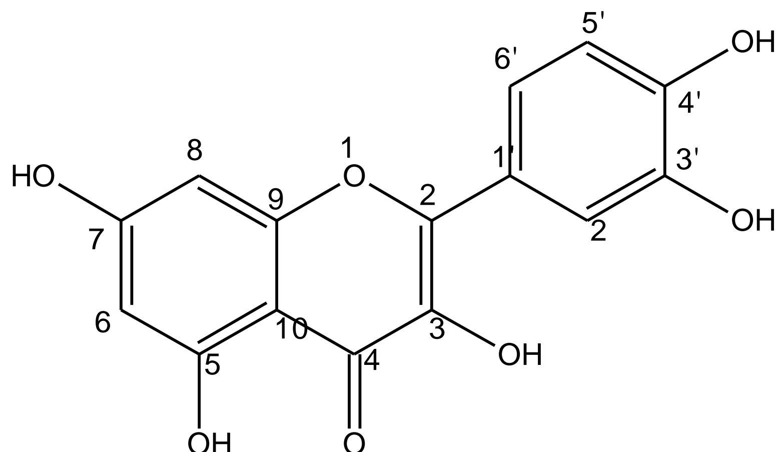


**Figure 5.** Fractions cp87-112 = 3,5,7,3’,4’-pentahydroxy flavone (quercetin).

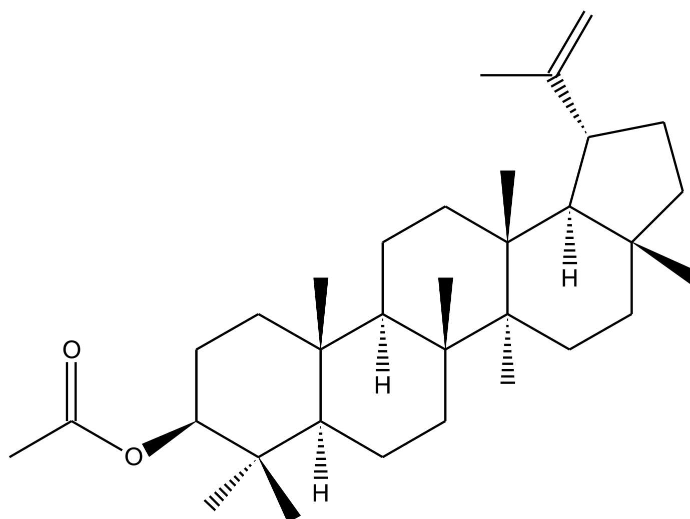


**Figure 6.** Fractions 73-91 LPE = Lupeol acetate.

H-5’), 7.30 (2H, m, H-2’ & H-6’), 5.34 (1H, d, J = 1.5 Hz, H-1’’), 3.90 (1H, dd, J = 1.5, 3.3 Hz, H-2’’), 3.72 (1H, m, H-3’’), 3.45 (1H, m, H-4’’), 4.18 (1H, m, H-5’’), 0.93 (3H, d, J = 6.0 Hz, H-6’’).

$^{13}$C NMR (MeOD, 300 MHz): δ 158.26 (C-2), 132.25 (C-3), 177.36 (C-4), 162.48 (C-5), 99.29 (C-6), 165.60 (C-7), 94.36 (C-8), 159.25 (C-9), 103.56 (C-10), 123.35 (C-1’), 116.72 (C-2’), 147.81 (C-3’), 147.18 (C-4’), 116.72 (C-5’), 122.93 (C-6’), 103.56 (C-2’’), 71.91 (C-3’’), 73.27 (C-4’’), 79.45 (C-5’’), 72.03 (C-6’’), 17.64 (C-7’’).

$^{1}$H NMR (Py, 300 MHz): δ 6.74 (1H, d, J = 2.2 Hz, H-6), 6.78 (1H, d, J = 2.2 Hz, H-8), 8.63 (1H, s, H-2’), 8.10 (1H, d, J = 8.4 Hz, H-6’), 7.40 (1H, d, J = 8.4 Hz, H-5’).

$^{13}$C NMR (Py, 300 MHz): δ 157.26 (C-2), 137.96 (C-3), 177.36 (C-4), 162.48 (C-5), 99.29 (C-6), 165.60 (C-7), 94.36 (C-8), 157.50 (C-9), 104.50 (C-10), 123.35 (C-1’), 116.72 (C-2’), 147.81 (C-3’), 147.18 (C-4’), 116.72 (C-5’), 121.12 (C-6’).

**Lupeol acetate: white needles (30 mg)**

$^{1}$H NMR ($CDCl_3$, 300 MHz): δ 4.69 (1H, *s*, H-29b), 4.57 (1H, *s*, H-29a), 4.47 (1H, *dd, J* = 4.4, 12.8 Hz, H-3), 2.05 (3H, *s*, H-2/), 1.69 (3H, *s*, H-30), 1.03 (3H, *s*, H-25) 0.94 (3H, *s*, H-28), 0.85 (3H, *s*, H-23), 0.84 (3H, *s*, H-24), 0.83 (3H, *s*, H-26), 0.79 (3H, *s*, H-27).

$^{13}$C NMR ($CDCl_3$, 300 MHz): δ 38.43 ($CH_2$,C-1), 27.83 ($CH_2$,C-2), 81.38 (CH,C-3), 38.78 (C-4), 55.77 (CH,C-5), 18.60 ($CH_2$,C-6), 34.60 ($CH_2$,C-7), 41.24 (CH,C-8), 50.73

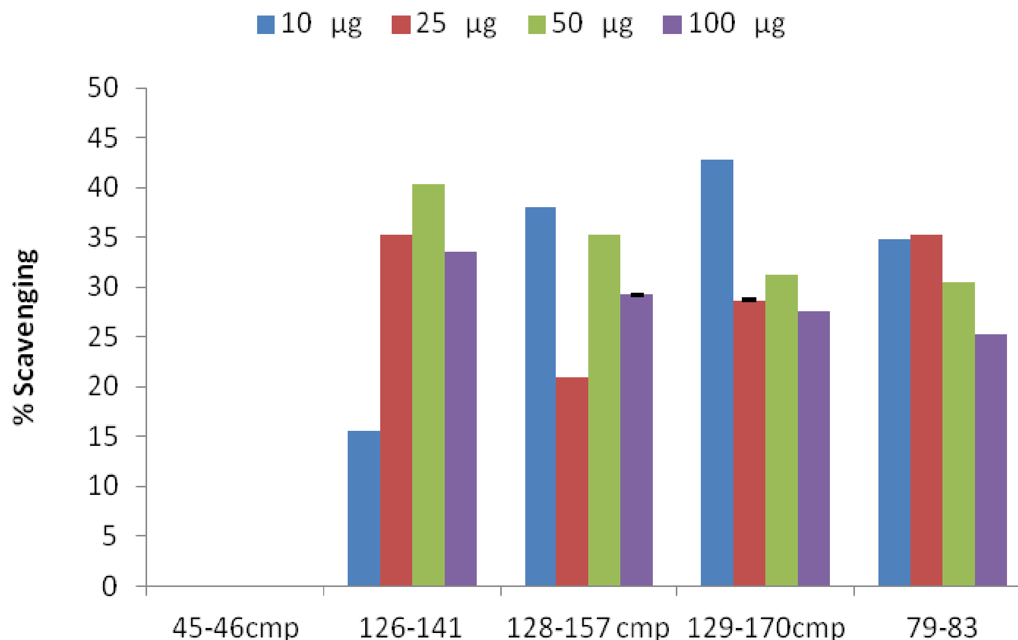


**Figure 7.** DPPH scavenging activity of pure compounds.

(CH,C-9), 37.48 (C-10), 21.33 ($CH_2$,C-11), 25.48 ($CH_2$,C-12), 38.19 (C-13), 43.22 (C-14), 27.83 ($CH_2$,C-15), 35.97 ($CH_2$,C-16), 43.40 (C-17), 48.67 (CH,C-18), 48.41 (CH,C-19), 151.37 (C-20), 30.11 ($CH_2$,C-21), 40.40 ($CH_2$,C-22), 28.35 ($CH_3$,C-23), 14.90 ($CH_3$,C-24), 16.59 ($CH_3$,C-25), 16.37 ($CH_3$,C-26), 14.54 ($CH_3$,C-27), 18.40 ($CH_3$,C-28), 109.77 ($CH_2$,C-29), 19.69 ($CH_3$,C-30), 171.45 (C-1’), 21.74 (C-2’).

Results of antioxidant studies are presented in Figures 7 to 13. In Figure 7, the amount of DPPH reduced was quantified by measuring increases in absorbance at 517 nm. The DPPH scavenging ability of the tested compounds may be attributed to their hydrogen donating ability. Non DPPH scavenging activity was observed for 73 to 91 LPE (Figure 6) and 45 to 46 cmp fractions (Figure 2). The fractions 126 to 141 (Figure 4), 128 to 157 cmp (Figure 3), cp87 to 112 (Figure 5) and 79 to 83 (Figure 1) have showed appreciable DPPH scavenging activity in different manner and for Figure 1 the activity decreases at 50 and 100 µg; this indicates that at higher concentration the activity was inhibited. This order was reversed in 126 to 141 fractions (Figure 4), in which the DPPH scavenging activity increases with increasing concentration of the compound but reduction was observed at 100 µg. Hence, the DPPH scavenging activity of the compounds may be represented as:

cp87 - 112 > 126 - 141 > 128 - 157 cmp > 79 - 83

Nitric oxides (NO) are potent inhibitors of physiological processes such as smooth muscle relaxation, neuronal signaling, platelet aggregation and regulation of cell mediated toxicity (Hagerman et al., 1998). In Figure 8, a non NO scavenging activities were observed for fractions 73 to 91 LPE (Figure 6). The nitric oxide (NO) scavenging activity of all the compounds was low with maximum of 22% inhibition for fractions 126 to 141 (Figure 4) at 25 µg but with no activity at 50 and 100 µg. The compound 45 to 46 cmp (Figure 2) has little NO scavenging activity but the activity increased as the concentration increased; however, the activity dropped drastically at 100 µg. Fractions 79 to 83 (Figure 1) did not show any activity. The compound 128 to 157 cmp (Figure 3) showed activity only at 10 µg, this shows that the NO scavenging activity of this compound was inhibited at concentration higher than 10 µg. The reducing power (Figure 9) of the compounds of fractions 126 to 141 (Figure 4), 128 to 157 cmp (Figure 3), and 79 to 83 (Figure 1) were found to increase in dose- dependent manner. Ferric reducing antioxidant power (FRAP) showed no activity in fractions cp87 to 112 (Figure 5) and 73 to 91 (Figure 6). However, 126 to 141 fractions (Figure 4) showed equal activity at 10 and 25 µg but there after increased dose - dependently. The 45 to 46 cmp fractions (Figure 2) did not show any activity. The ferric reducing antioxidant power (FRAP assay) is widely used in the evaluation of the antioxidant components of polyphenols (Luximan-Ramma et al., 2005). From the results, there is a relationship between total phenols and reducing power of the tested pure compounds. The FRAP assay (Figure 13) may be represented as:

79 - 83 > 128 - 157 cmp > 126 - 141.

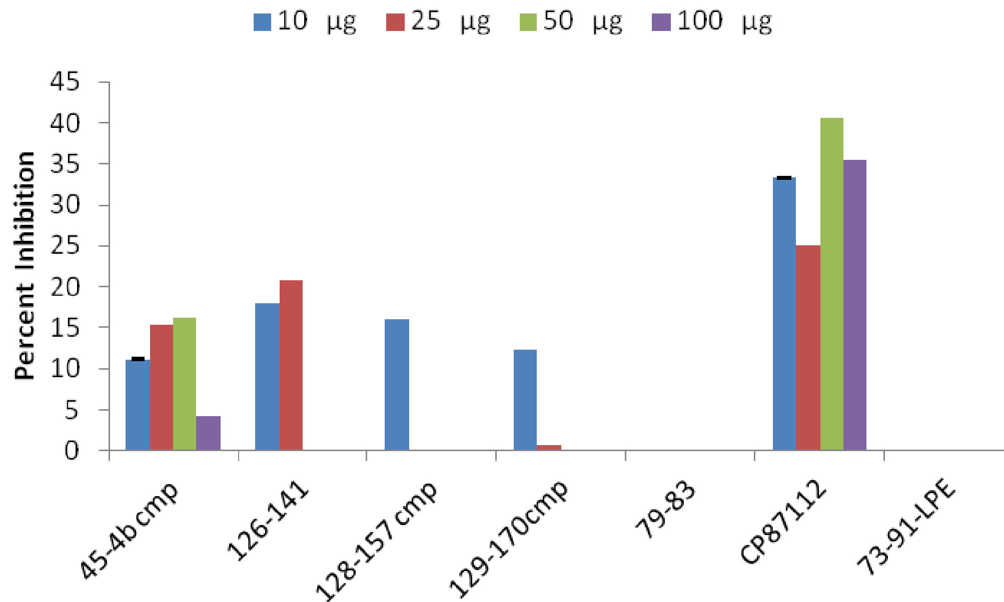


**Figure 8.** Nitric oxide scavenging activity of pure compounds.

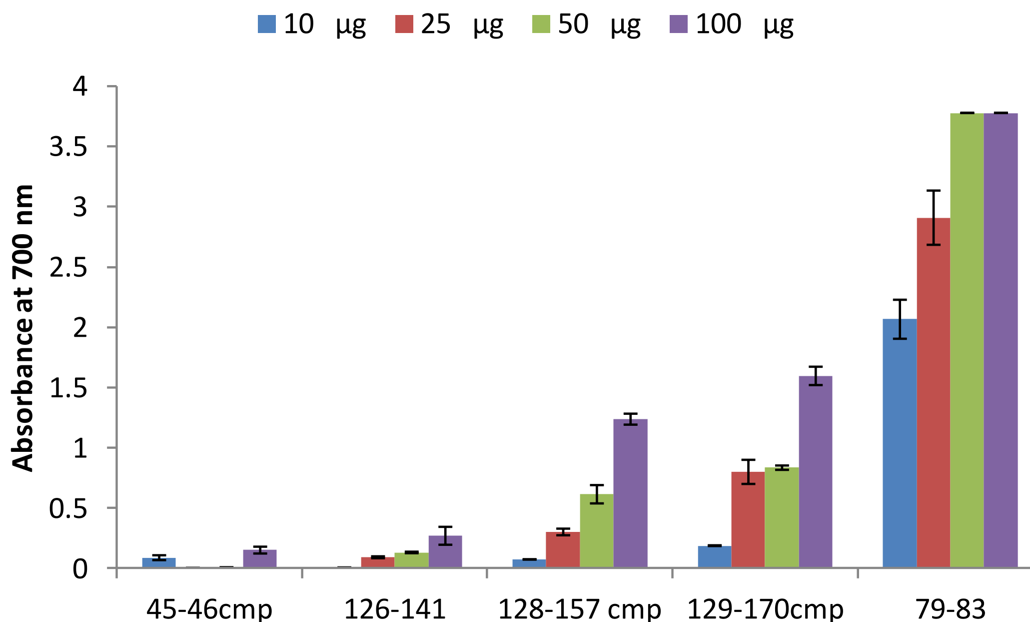


**Figure 9.** Reducing power ability of pure compounds.

In Figure 10, little amount of total flavonoids (TF) were observed for 73 to 91 LPE fractions (Figure 6). The total flavonoids contents were also found to increase in a dose- dependent manner for 128 to 157 cmp (Figure 3) and 79 to 83 (Figure 1) compounds. It dropped slightly at 100 µg for compound 126 to 141 (Figure 4) and at 50 to 100 µg for 45 to 46 cmp (Figure 2). The total phenolics (TP) were increased in dose dependant manner with exception of 45 to 46 cmp fractions (Figure 2) and 73 to 91 LPE (Figure 6) that showed little or no activity (Figure 11). The antioxidant activity of polyphenolic compounds is mainly due to their redox properties which play an important role in adsorbing to and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Zheng and Wang, 2001). Phytochemicals like polyphenols possess significant antioxidant

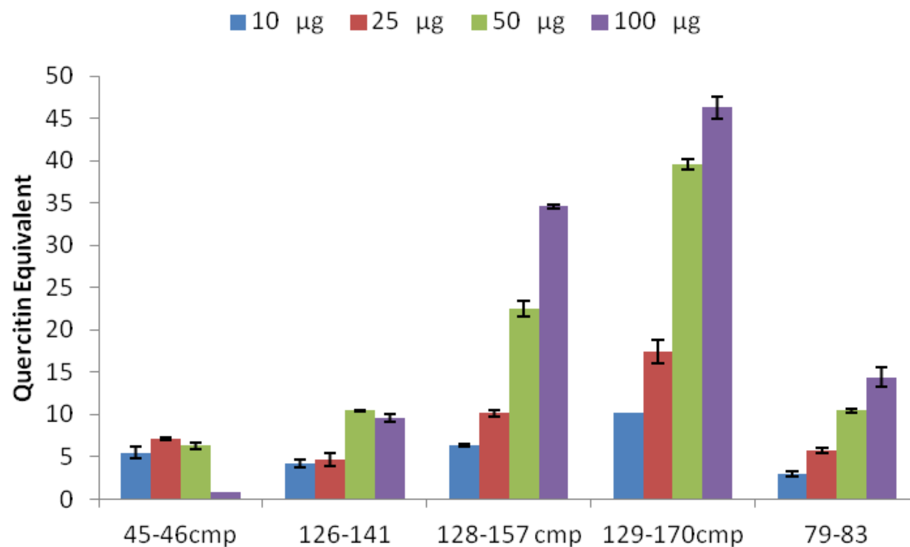


**Figure 10.** Total flavonoids capacity in terms of quercetin equivalent of pure compounds.

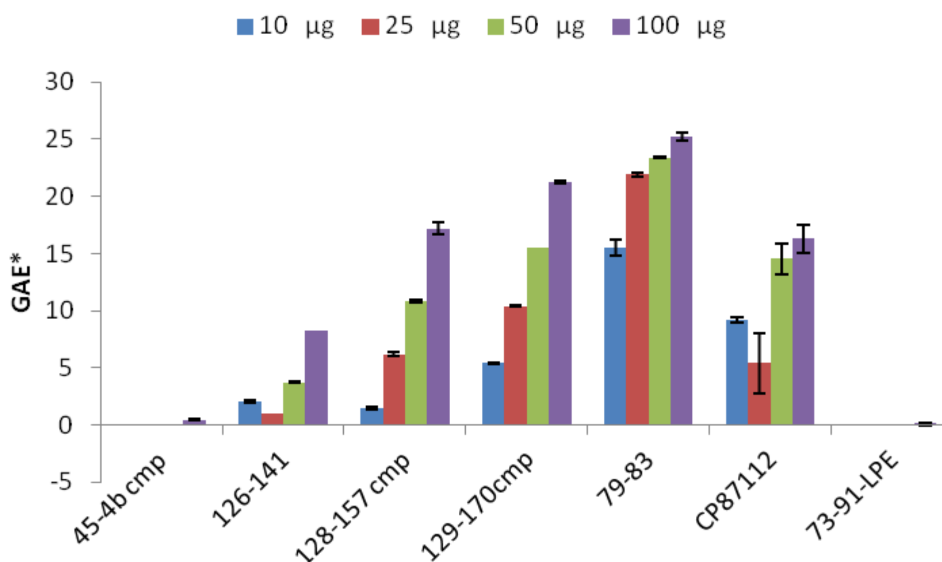


**Figure 11.** Total phenolics expressed in terms of Gallic acid equivalent (GAE*) of pure compounds.

capacities that are associated with lower mortality and rate of diseases (Anderson et al., 2001; Djeridane et al., 2006). The pharmacological effect demonstrated by the ethylacetate fraction of the combined plants mixture suggests that the phenolics have some pharmacological effects and could be attributed to these valuable constituents. All the results of total phenolic estimation were expressed as Gallic acid equivalent (Figure 11) and are represented as follows:

79 - 83>128 - 157 cmp > 126 - 141 > 45 - 46 cmp

For total antioxidant capacity (TAC), 73 to 91 LPE fractions (Figure 6) did not show any activity. TAC in 45 to 46 cmp fractions (Figure 2), 128 to 157 cmp (Figure 3) were found to increase in a dose- dependent manner but for 126 to 141 (Figure 4) it increases from dose 25 to 100 µg. However, 79 to 83 (Figure 1) showed only equal activity at 10 and 25 µg. All the results were expressed as ascorbate equivalent (Figure 12) and may also be represented as follows:

45 - 46 cmp > 128 - 157 cmp > 79 - 83 > 126 - 141

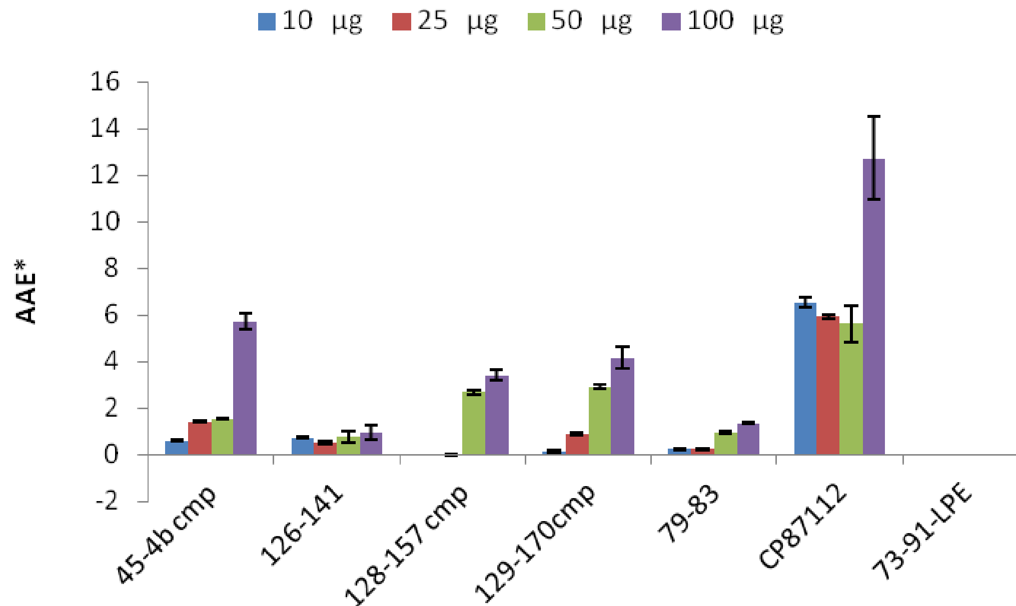


**Figure 12.** Total antioxidant capacity in terms of ascorbate equivalent (AAE*) of pure compounds.

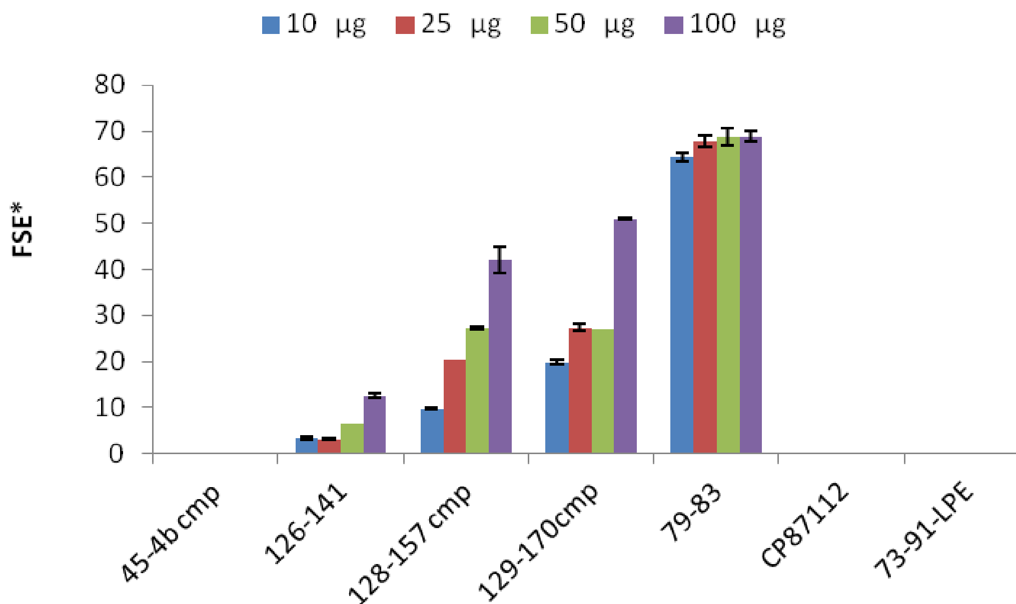


**Figure 13.** FRAP assay expressed in terms of ferrous sulphate equivalent (FSE*) of pure compounds.

The effects of the isolated compounds in the present study is due to their phenolic acids and flavonoids nature and have been demonstrated to exhibit antioxidant activity (Sliva et al., 2006; Kasture et al., 2009).

## Conclusion

These findings, suggest that the extracts/pure compounds possess antibacterial and antioxidant properties. The pharmacological effects demonstrated by the extracts could be attributed to their phytocompounds. Further screenings for in vitro antimalarial and anticancer activities of the compounds isolated are recommended.

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*Full Length Research Paper*

# Immobilization of the *Candida rugosa* lipase onto a *Scirpus grossus* L.f. fiber as biocatalyst for biodiesel synthesis via hydrolysis-esterification

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**This study aimed to immobilize the *Candida rugosa* lipase (*C. rugosa* lipase) on ground *Scirpus grossus* L.f. fibers by glutaraldehyde-crosslinking to form a hydrolysis-esterification catalyst for biodiesel synthesis. The effects of different glutaraldehyde concentrations and solvent for 3-aminopropyltriethoxysilane (3-APTES) activation of the fibers on the resultant immobilized lipase activity, protein loading, degree of immobilization and catalytic efficiency were investigated. The optimum condition found was to first activate the *S. grossus* L.f. fibers using 2% (w/v) of 3-APTES in distilled water and subsequently crosslink with 0.2% (w/v) glutaraldehyde prior to *C. rugosa* lipase immobilization at pH 7. The immobilized *C. rugosa* lipase was then evaluated as a biocatalyst for biodiesel synthesis via the hydrolysis-esterification of palm oil and bioethanol through monitoring the production of free fatty acids (FFAs) and fatty acid ethyl ester (FAEE, biodiesel). The reusability of immobilized lipase was also determined. The immobilized *C. rugosa* lipase yielded a higher hydrolysis-esterification efficiency (that is, FFA and FAEE formation) than that of the free lipase with the immobilized form looking promising for FAEE biodiesel production. The *C. rugosa* lipase immobilized with 0.2% (w/v) glutaraldehyde exhibited the highest reuse stability, retaining some hydrolysis and esterification activity for up to six uses, whereas crosslinking with higher [0.5% or 0.8% (w/v)] glutaraldehyde levels resulted in a loss of both activities within four uses.**

**Key words:** Immobilization, lipase, *Scirpus grossus* L.f. fiber, 3-aminopropyltriethoxysilane, glutaraldehyde, biodiesel.

## INTRODUCTION

Currently, the limited petroleum-based oil reserves, the increase of oil price due to the decrease of crude oil production because of the political problem of the middle-east countries, and the environmental concerns from $CO_2$ emission have urged many researchers to develop alternative renewable energy sources. Biodiesel is viewed as one of the new potential alternative renewable, non-toxic, biodegradable and clean energy sources for

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petroleum-based fuel replacement (Gerpen, 2005). It is claimed as a nonpolluting fuel because the burning of biodiesel does not contribute to global warming due to its large reduction of $CO_2$ emission compare with petroleum-based oil. Although, biodiesel can be successfully produced by chemical catalysis, this is a high energy consumption process and the recovery of the biodiesel from the glycerol byproduct is difficult. Moreover, this process also produces large amounts (ca. 10% of the volume of biodiesel produced) of non-environmentally friendly wastewater that is difficult to treat and also expensive. Recently, the use of lipase as biocatalyst instead of acid or alkali catalysts has attracted considerable attention because byproduct glycerol can be recovered easily and the purification of the biodiesel (fatty acid methyl esters, FAME) is simple to accomplish. In addition, this process is non-toxic, has a lower energy requirement, and also has biodegradable characteristics (Shimada et al., 1999; Nelson et al., 1996). However, the high cost of lipase makes enzymatic processes commercially unattractive. Therefore, immobilization of lipase has advantages over free lipase in terms of its reusability, its ease of removal from the reaction mixture and its adaptability to various engineering designs (Xie and Ma, 2009). Nevertheless, the high cost of com-mercial immobilized lipase due in part to the requirement for expensive support materials, makes production of biodiesel by this approach more expensive than that produced by chemical catalysis and so it is not economically viable. Thus, cheaper supports such as clays, silica gels and glasses have been evaluated as alternative adsorbents to the expensive polymeric resins (Hung et al., 2003; Minovska et al., 2005; Lee et al., 2006; Sugunan et al., 2007).

Other attractive supports are plant fibers that are composed of cellulose because of their accessibility, cheapness, hydrophilic nature and the great number of hydroxyl groups on the surface that are capable of chemical reaction (Przybyt and Sugier, 1988). In addition, most of them possess a large number of pores, allowing the lipase to be immobilized onto the plant fibers through simple adsorption. *Scirpus grossus* L.f. or *Cyperus papyrus* L., is an abundant biomass cellulose fiber in Thailand. Due to its very low cost, lack of toxicity and its chemical reactivity that allows it to react with the enzyme easily, *S. grossus* L.f. fibers are viewed as a non-toxic, economic and environmental friendly support for lipase immobilization. Lipase immobilization onto *S. grossus* L.f. fibers by physical adsorption is simple but appears to be that they cannot maintain their active configuration and so lose catalytic activity with time for biodiesel production via transesterification. Lee et al. (2006) developed a suitable method for the immobilization of *Rhizopus oryzae* lipase on silica gel using glutaraldehyde as a crosslinking agent, reporting on the optimizing of each step of the immobilization procedures, such as the pretreatment of silica gel, silanization, crosslinking and glutaraldehyde modification.

Silanization on the silica gel surface using 3-aminopropyltriethoxysilane (3-APTES) exhibited the highest resultant catalytic activity of the immobilized lipase. Furthermore, after 20 consecutive uses in a 30 min reaction with repeated washing between each reaction, some 80% of the enzyme activity of the immobilized lipase was still retained. Wang et al. (2008) reported that the immobilization of *Candida rugosa* lipase (*C. rugosa* lipase) onto the dense surface of a polysulfone ultrafiltration membrane by filtration and then crosslinking with glutaraldehyde solution greatly improved the lifetime of the enzyme. The apparent reaction rate and stability of the immobilized lipase were greatly improved after crosslinking. Xie and Ma (2009) reported that immobilized lipase on $Fe_3O_4$ nanoparticles treated with 3-APTES was using as biocatalyst for biodiesel production via transesterification. The conversion of soybean oil to biodiesel fuels reached over 90% by the three-step addition of methanol when 60% immobilized lipase was employed. In addition, it could be used four times without significant decrease of activity. Thus, to reduce the hydrophilicity of *S. grossus* L.f. and enhance the stability and retain a high catalytic activity of the immobilized lipase, it is necessary to modify or activate the surface properties of cellulosic fibers enriched with hydroxyl groups using a silane coupling agent and subsequent crosslinking of the lipase to the support material using glutaraldehyde.

In this study, glutaraldehyde-crosslinking based immobilization of the *C. rugosa* lipase onto 3-APTES activated ground *S. grossus* L.f. fiber was investigated for its suitability for biodiesel synthesis. Protein loading, lipase activity and degree of immobilization of immobilized the *C. rugosa* lipase were subsequently determined. The catalytic activities of the immobilized *C. rugosa* lipase in a hydrolysis-esterification reaction using palm oil and bioethanol for FAEE biodiesel synthesis was also examined to ascertain its biocatalyst potential including the reusability of the immobilized *C. rugosa* lipase.

## MATERIALS AND METHODS

*C. rugosa* lipase powder (EC 3.1.1.3) was purchased from Sigma-Aldrich (USA). Plant fibers of *S. grossus* L.f. from Nakhon Ratchasima Province (Thailand) were used as the support. They were dried and ground into fine powder using a Pulverizer model T15 before use. 3-APTES used for the activation of hydroxyl group of *S. grossus* L.f. and glutaraldehyde (50% in water) used as a crosslinking agent, were purchased from Fluka (Switzerland). Palm oil was obtained from Morakot Industries (Thailand) and used as the substrate triglyceride. Bioethanol was kindly provided by the Petroleum Authority of Thailand.

### *C. rugosa* lipase immobilization

The procedure for *C. rugosa* lipase immobilization was composed

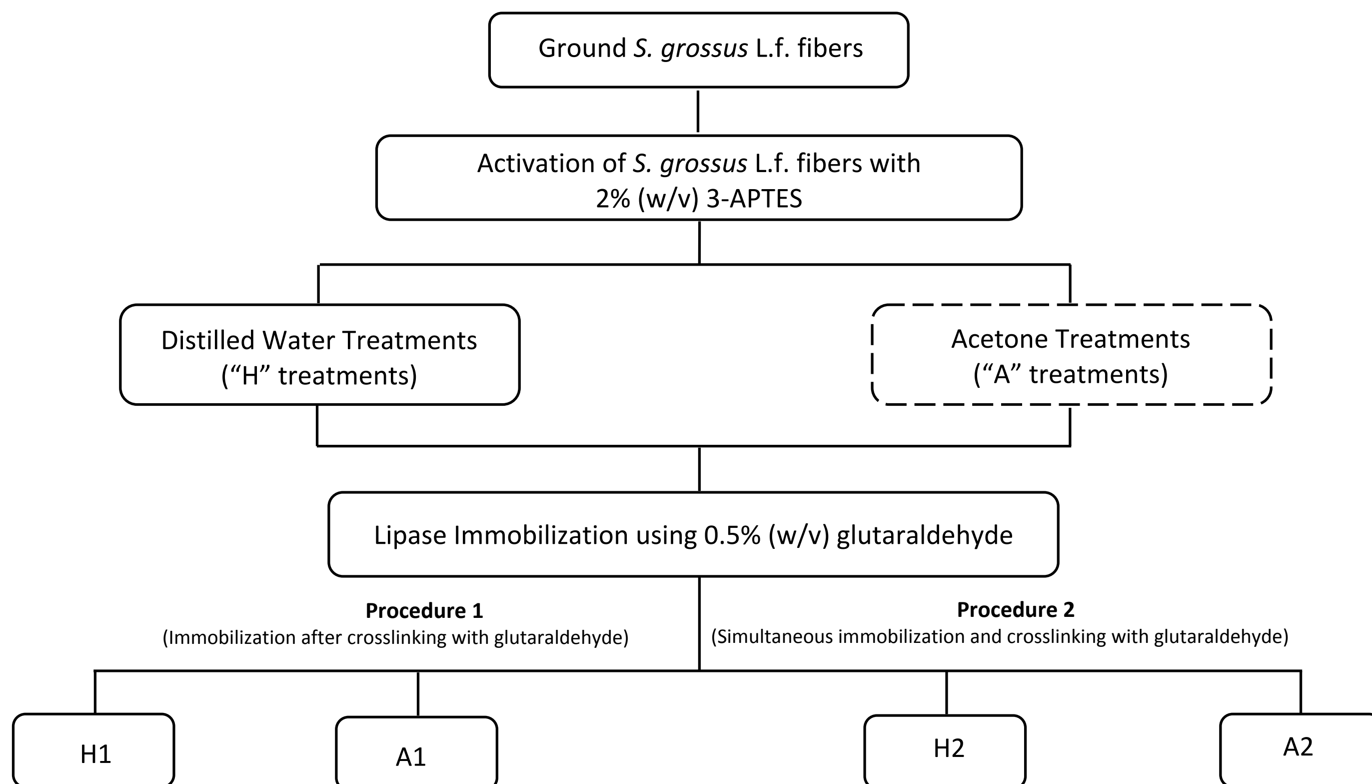



**Figure 1.** Flow diagram of *C. rugosa* lipase immobilization onto ground *S. grossus* L. f. fibers.

of two main steps. The first step was to activate the ground fiber using 3-APTES solution in either distilled water (H treatment) or in acetone (A treatment). The second step was to immobilize the *C. rugosa* lipase before crosslinking with glutaraldehyde (A1 and H1 treatments) or to simultaneously immobilize and crosslink the lipase onto the fibers with glutaraldehyde (A2 and H2 treatments), as detailed below. The flow diagram of the *C. rugosa* lipase immobilization is shown in Figure 1.

### *Activation of S. grossus* **L.f. fibers** *with 3-APTES in distilled water (H treatments) or in acetone (A treatments)*

Five grams of ground *S. grossus* L.f. fibers was mixed with 100 ml of 2% (w/v) of 3-APTES solution in either distilled water or in acetone and stirred at 360 rpm for 30 min. The activated ground fiber was then washed with distilled water three times and left to dry at 80°C for 6 h.

### *Lipase immobilization using glutaraldehyde as a crosslinking agent*

***Procedure 1* (immobilization after crosslinking):** One gram of the H or A activated ground *S. grossus* L.f. fibers crosslinked with 20 ml of 0.5% (w/v) glutaraldehyde in 0.05 M phosphate buffer (pH 7). The reaction mixture was stirred at 360 rpm for 1 h at room temperature and then washed with distilled water three times, vacuum filtered, left to dry and kept in a desiccator. Thereafter, 0.5 g of the crosslinked-ground fiber was mixed with 10 ml of a 0.01 g/ml *C. rugosa* lipase solution in 1 M phosphate buffer (pH 7) and stirred at 360 rpm for 12 h at room temperature. The immobilized lipase was then washed six times, each with 10 ml of 0.05 M phosphate buffer solution (pH 7), vacuum filtered and left to dry in a desiccator for overnight prior to storage at 4°C.

***Procedure 2* (simultaneous immobilization and crosslinking):** To 0.5 g of A or H treated ground, *S. grossus* L.f. fibers was mixed with 10 ml of 0.01 g/ml *C. rugosa* lipase solution in 1 M phosphate buffer (pH 7), and 100 μl of glutaraldehyde [at 0.2, 0.5 or 0.8% (w/v)] was then added and stirred at 360 rpm for 12 h at room temperature. The immobilized lipase was then washed six times, each with 10 ml of 0.05 M phosphate buffer (pH 7), vacuum filtered, dried and kept in a desiccator overnight and stored at 4°C.

### Morphology of immobilized lipase

Scanning Electron Microscopy (SEM), using a Jeol JSM-5800 LV electron microscope (Japan) under an accelerated voltage of 15 KV, was performed at a magnification of 750x to examine the morphology of the ground *S. grossus* L.f. fibers and the adsorption of the *C. rugosa* lipase to the fiber support after immobilization.

### Determination of *C. rugosa* lipase activity

The activity of the free and immobilized lipase was determined using 0.5% (w/v) *p*-nitrophenyl palmitate (*p*-NPP) in ethanol as the substrate. The increase in absorbance at 410 nm caused by the

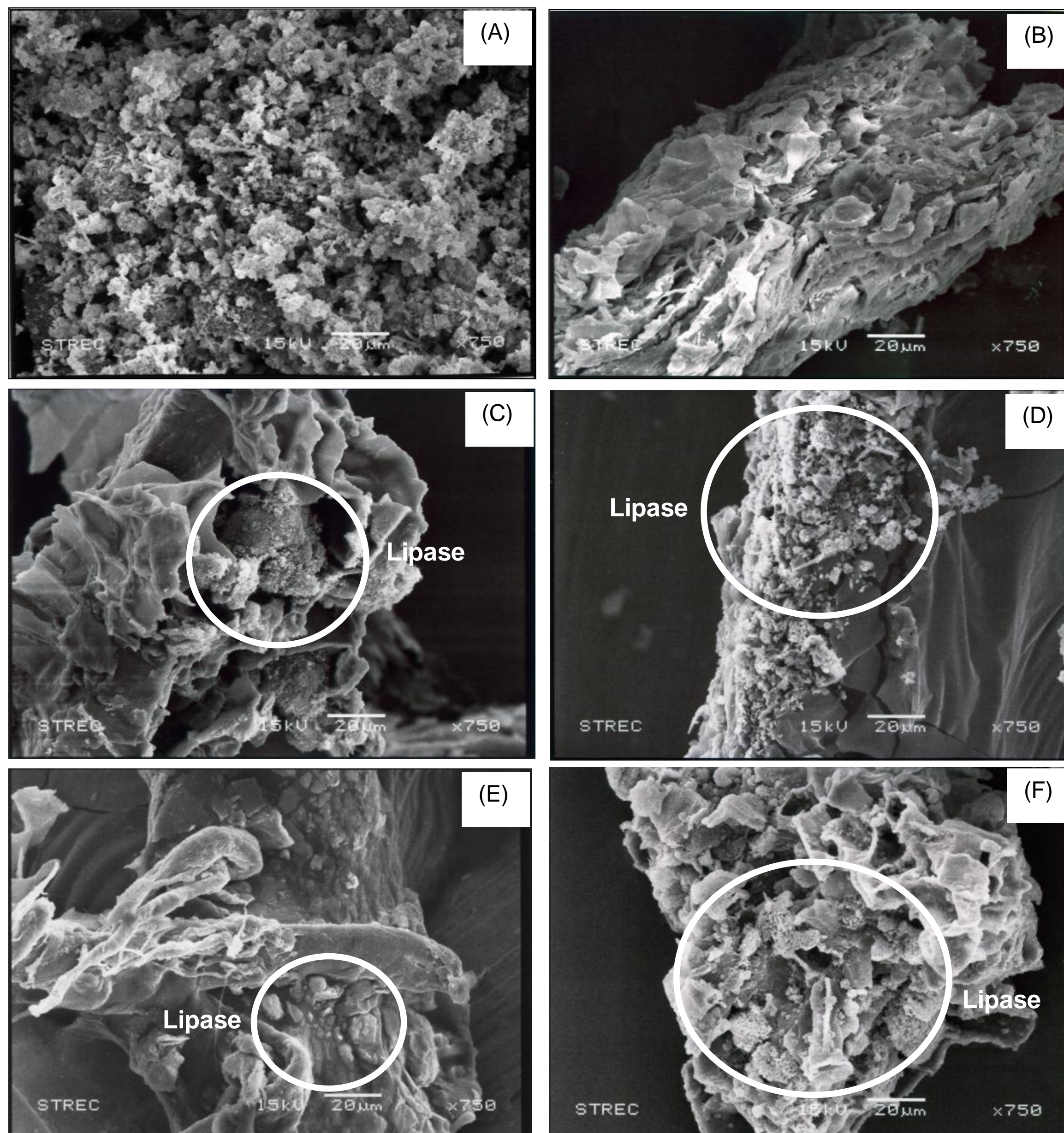



**Figure 2.** Representative SEM images of the (A) free lipase, (B) fine ground powder of *S. grossus* L.f. fibers, (C-F) immobilized lipase on the *S. grossus* L.f. fibers after activation with 3-APTES (C,D) in distilled water or (E,F) in acetone and crosslinking with 0.5% (w/v) glutaraldehyde either (C,E) after or (D,F) simultaneous lipase immobilization.

release of *p*-nitrophenol, was measured with a UV spectrophotometer (ANTHOS Zenyth 200 microplate spectrophotometer). One unit (U) of lipase activity was defined as the amount of enzyme required to hydrolyze 1 nmol/min of *p*-NPP under the experimental conditions.

### Determination of protein loading

Protein concentration was spectrophotometrically determined with a UV spectrophotometer (ANTHOS Zenyth 200 Microplate Spectrophotometer) according to the Bradford method using bovine serum albumin (BSA) as the standard (Bradford, 1976). The protein loading level is shown as the amount of lipase incorporated in the fibers as a percentage of the total amount added.

### Determination of immobilization efficiency

The efficiency of each immobilization technique, $\eta$, was estimated by calculating the activity of the lipase solution before ($E_0$) and after ($E_f$) immobilization, using Equation 1:

$\eta = (E_0V_0 - E_fV_f)/E_0V_0 \times 100$ (1)

Where, $V_0$ is the initial volume of lipase solution (ml) and $V_f$ is the total volume of lipase solution after filtration (ml). The activities are given in U/ml.

**Biodiesel synthesis**

In this study, the catalytic activities of the immobilized *C. rugosa* lipase for the hydrolysis-esterification reaction of palm oil and bioethanol as substrates for FAEE biodiesel synthesis were studied through the monitoring of the free fatty acid (FFA) and FAEE production levels as follows:

***Hydrolysis of palm oil***

Palm oil was first hydrolyzed by mixing 3 g of palm oil and 10% (of the weight of palm oil) of the immobilized *C. rugosa* lipase suspension or the free *C. rugosa* lipase solution. Then, 300 µl of distilled water was added to the mixture and continuously stirred at 600 rpm for 12 h at 50°C. From this, 100 µl of the hydrolytic product was removed and centrifuged at 13,000 rpm for 30 min. The upper phase was used to quantify the FFA composition using high performance liquid chromatography (HPLC) with a, Shimadzu LC-20A series (Japan).

***Esterification***

The stepwise ethanolysis of palm oil was conducted using a 1:3 molar ratio of palm oil:bioethanol as this ratio has been reported to be optimal (Shimada et al., 1999). However, the bioethanol was added to the hydrolyzed palm oil in three stages feeding of bioethanol at each of 0, 8 and 16 h into palm oil and stirring at 600 rpm for 24 h at 50°C. At completion, 100 µl of the product was removed and centrifuged at 13,000 rpm for 30 min. The upper phase was used to quantify the FFA and FAEE composition by HPLC as described earlier.

**Evaluation of the reusability of immobilized *C. rugosa* lipase**

After being used as a catalyst for the hydrolysis-esterification, the immobilized *C. rugosa* lipase was filtered, washed with 20 ml heptane and store in a desiccator for 12 h prior to reuse for the next batch reaction. The conversion of palm oil / bioethanol to FFA and FAEE at the end of the reaction was evaluated by HPLC as aforementioned. This process was repeated until no significant production of FFA or FAEE could not be detected.

## RESULTS AND DISCUSSION

### Morphology of the immobilized *C. rugosa* lipase on *S. grossus* L.f. fibers

Representative SEM images of the free lipase, ground *S. grossus* L.f. fibers and the 0.5% (w/v) glutaraldehyde crosslinked and immobilized *C. rugosa* lipase on *S. grossus* L.f. fibers prepared by different procedures are shown in Figure 2. The presence of lipase particles was seen on the surface and into the pores of the fiber support prepared by all procedures. The activation of the ground *S. grossus* L.f. fibers support with 3-APTES solution in water (H1 and H2) rendered a higher level of lipase adsorption onto the surface and into the pores of the fiber support than the 3-APTES activation in acetone (A1 and A2). In addition, the lipase adsorption via the simultaneous immobilization and crosslinking procedure revealed a slightly higher lipase adsorption level than the separate immobilization of the lipase after crosslinking.

This can be attributed to: (i) the use of an aqueous swelling medium that favored the diffusion of the coupling agents (3-APTES) into the pores of the cellulose substrate and consequently increased the available interaction area and (ii) the presence of the $NH_2$ groups in the 3-APTES favored the interaction between the coupling agents and the cellulose substrate (Abdelmouleh et al., 2002).

### Degree of immobilization and catalytic efficiency

The use of four different procedures to prepare immobilized lipase, which are the sequential or simultaneous immobilization and crosslinking in water or acetone (H1, H2, A1 and A2), yielded different final lipase activities, protein loading levels, and degree of immobilization and the catalytic efficiencies (Table 1). The highest lipase activity, protein loading and degree of immobilization of the immobilized lipase was achieved by first activating the *S. grossus* L.f. fibers with 3-APTES (in distilled water) and then immobilization of the lipase after crosslinking with glutaraldehyde (treatment H1; Table 1). However, with respect to the catalytic efficiency, defined as the ratio between the lipase activity and the protein loading level, this method gave a marginally lower catalytic efficiency than when 3-APTES activated in acetone (A1 in Table 1). The higher the catalytic efficiency, the lower the amount of lipase that has been inactivated by the immobilization (Salis et al., 2008).

A higher catalytic efficiency of the immobilized lipase was observed when it was prepared by immobilization after crosslinking rather than when it was simultaneously immobilized and crosslinked regardless of the APTES treatment was in water or acetone (compare treatments A1 with A2 and H1 with H2; Table 1). This is presumed to be because the unreacted aldehyde groups of glutaraldehyde remain after crosslinking with an amino group of the lipase and so can attach to the other amino groups of the lipase, distorting the conformation and so reduce lipase's activity (Lee et al., 2006). In addition, simultaneous immobilization and crosslinking may lead to encourage binding between glutaradehyde and lipase. This may influence to the active site of enzyme and reduce its catalytic efficiency. From these results, the immobilized *C. rugosa* lipase prepared by first activating the ground *S. grossus* L.f. fibers with 3-APTES in distilled water and subsequentially immobilizing the *C. rugosa* lipase after crosslinking with glutaraldehyde (treatment

**Table 1.** Lipase activity, protein loading, degree of immobilization and catalytic efficiency of the immobilized *C. rugosa* lipase prepared by activation of *S. grossus* L.f. fibers with 3-APTES in either distilled water (H) or acetone (A) and then either sequential (treatments H1 and A1) or simultaneous (treatments H2 and A2) immobilization after crosslinking with 0.5% (w/v) glutaraldehyde.

| Technique of immobilization | Lipase activity (U/g-fiber) | Protein loading (%) | Degree of immobilization (%) | Catalytic efficiency[a] |
|---|---|---|---|---|
| H1 | 3.31 | 79.6 | 84.2 | 0.042 |
| H2 | 2.73 | 70.5 | 69.5 | 0.039 |
| A1 | 3.22 | 74.4 | 81.9 | 0.043 |
| A2 | 3.03 | 78.7 | 77.0 | 0.039 |

[a]Catalytic efficiency is the ratio of the lipase activity to the protein loading level.

**Table 2.** Effects of varying the glutaraldehyde concentration on the resulting immobilized lipase activity, protein loading, degree of immobilization and catalytic efficiency of the immobilized *C. rugosa* lipase. The immobilized *C. rugosa* lipase was prepared by activation of *S. grossus* L.f. fibers with 3-APTES in distilled water and immobilized after glutaraldehyde crosslinking (treatment H1).

| Glutaraldehyde concentrations [% (w/v)] | Lipase activity (U/g-fiber) | Protein loading (%) | Degree of immobilization (%) | Catalytic efficiency[a] |
|---|---|---|---|---|
| 0.2 | 3.01 | 80.4 | 76.6 | 0.037 |
| 0.5 | 3.31 | 79.6 | 84.2 | 0.042 |
| 0.8 | 2.93 | 77.7 | 74.5 | 0.038 |

[a]Catalytic efficiency is the ratio of the lipase activity to the protein loading level.

(treatment H1) was further investigated. Under this partially optimized condition, the effects of varying the glutaraldehyde concentrations and pH on the degree of enzyme immobilization and hydrolysis-esterification catalytic activity in the FAEE biodiesel synthesis from palm oil and bioethanol was evaluated next.

### Effects of the glutaraldehyde concentrations on the degree of *C. rugosa* lipase immobilization and hydrolysis-esterification catalytic efficiency

The effects of varying the glutaraldehyde concentrations on the resultant lipase activity, protein loading, degree of immobilization and catalytic efficiency of the immobilized *C. rugosa* lipase were evaluated. Immobilized *C. rugosa* lipase was prepared by activation of *S. grossus* L.f. fibers with 3-APTES in distilled water crosslinking with glutaraldehyde at 0.2, 0.5 or 0.8% (w/v) and then the lipase was immobilized (treatment H1).

The highest lipase activity, degree of immobilization and catalytic efficiency were achieved with 0.5% (w/v) glutaraldehyde, although a slightly higher protein loading level was obtained with 0.2% (w/v) glutaraldehyde (Table 2). Too low a glutaraldehyde concentration did not immobilize the lipase enough to be stably held on the fiber surface. In contrast, too high glutaraldehyde concentration would damage the active sites of the lipase (Wang et al., 2008).

### Effects of pH on the degree of *C. rugosa* lipase immobilization and hydrolysis-esterification catalytic efficiency

The effects of pH on the resultant lipase activity, protein loading, degree of immobilization and catalytic efficiency of the immobilized *C. rugosa* lipase were shown in Table 3. Immobilized *C. rugosa* lipase was prepared by activation of *S. grossus* L.f. fibers with 3-APTES in distilled water crosslinking with 0.2% (w/v) glutaraldehyde and then the lipase was immobilized (treatment H1) at pH 5, 7 and 9. The highest lipase activity, degree of immobilization were achieved at pH 7, whereas, the catalytic efficiency at pH 5 and 7 were similar. Thus, the pH optimum for the *C. rugosa* lipase immobilization was pH 7 or mild pH 5 rather than pH 9. This may be the bond between lipase and the support is unstable in basic reaction media (pH > 7) and stable in acid and neutral media (pH 5 and 7).

### Production of FFAs and FAEE biodiesel from palm oil and bioethanol

The % conversion of palm oil to FFA (hydrolysis activity) and subsequently with the bioethanol to FAEE (esterification activity) is summarized in Table 4. The three immobilized *C. rugosa* lipase preparations all yielded a higher FFA and especially and importantly,

**Table 3.** Effects of pH on lipase activity, protein loading, degree of immobilization and catalytic efficiency of the immobilized *C. rugosa* lipase. The immobilized *C. rugosa* lipase was prepared by activation of *S. grossus* L.f. fibers with 3-APTES in distilled water and immobilized after crosslinking with 0.2% (w/v) glutaraldehyde (treatment H1).

| pH | Lipase activity (U/g-fiber) | Protein loading (%) | Degree of immobilization (%) | Catalytic efficiency[a] |
|---|---|---|---|---|
| 5 | 2.77 | 72.7 | 76.6 | 0.038 |
| 7 | 3.01 | 80.4 | 84.2 | 0.037 |
| 9 | 2.16 | 63.2 | 74.5 | 0.034 |

[a]Catalytic efficiency is the ratio of the lipase activity to the protein loading level.

**Table 4.** Conversion of free fatty acid (FFA) and fatty acid ethyl ester (FAEE; biodiesel) produced by the immobilized *C. rugosa* lipase from a 3:1 molar ratio of palm oil: bioethanol. The immobilized *C. rugosa* lipase was prepared by activation of *S. grossus* L.f. fibers with 3-APTES in distilled water and immobilized after glutaraldehyde crosslinking at various concentrations (treatment H1).

| Immobilized *C. rugosa* lipase [% (w/v) glutaraldehyde] | Free fatty acid (FFA) (%) | Fatty acid ethyl ester (FAEE) (%) |
|---|---|---|
| Free lipase | 41.6 | 14.7 |
| 0.2 | 52.6 | 49.6 |
| 0.5 | 43.9 | 32.2 |
| 0.8 | 43.5 | 23.7 |

FAEE production level than that of the free *C. rugosa* lipase. The immobilized *C. rugosa* lipase could catalyze the hydrolysis and esterification reaction relatively efficiently.

Minovska et al. (2005) reported previously that the free enzyme must be used at three-times higher quantities than the immobilized lipase, whilst the free lipase may lose its enzymatic activity under severe environments and with a lower surface area to catalyze the reaction. Of the immobilized *C. rugosa* lipase preparations tested here, that produced by crosslinking with 0.2% (w/v) glutaraldehyde provided the highest levels of FFA and FAEE, and so the highest hydrolysis-esterification catalytic activity. Too high concentrations of glutaraldehyde [0.8% (w/v)] gave lower yields, presumably due to the strong covalent binding of the lipase and glutaraldehyde multiple sites causing the enzyme denaturation and loss of some active sites of the lipase.

## Reusability of immobilized enzyme for biodiesel synthesis

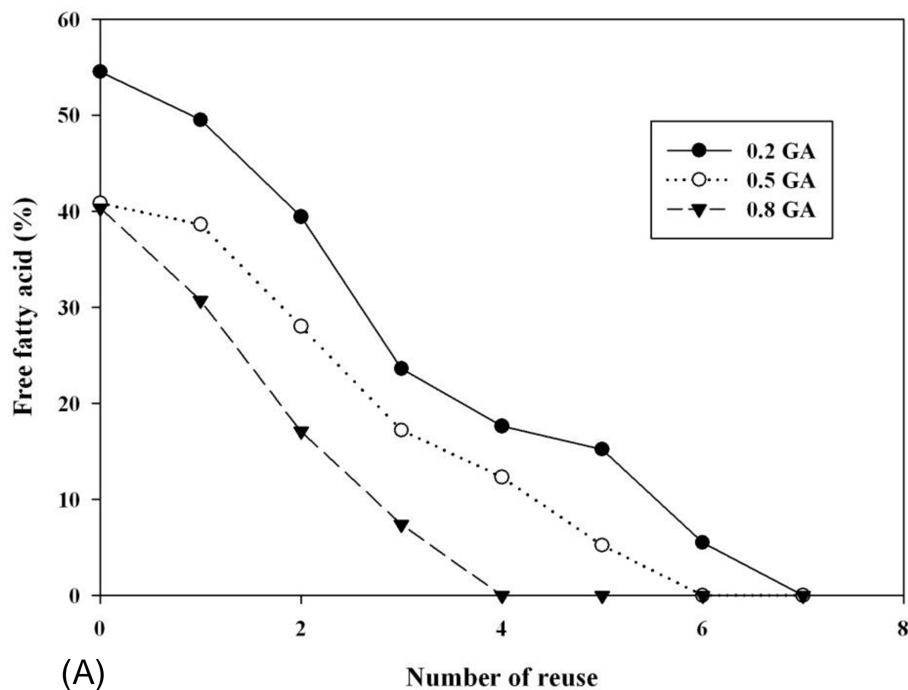
The stability of the immobilized lipase is important for any subsequent biodiesel synthesis. Therefore, the long-term stability of the prepared immobilized lipase after biodiesel synthesis was evaluated. The immobilized lipase activity declined after each use for both the hydrolysis (FFA production; Figure 3A) and esterification (FAEE production; Figure 3B) catalytic activities. However, note that the esterification activity is dependent upon the availability of FFA and so a reduction in the hydrolysis efficiency may be manifest as an apparent but false reduction in the esterification catalytic efficiency when FFA levels are low.

The lipase immobilized with 0.2% (w/v) glutaraldehyde exhibited the highest reuse stability with some 50% residual FFA and FAEE levels after three or one reuse, respectively, and with some hydrolysis-esterification activity remaining at up to six reuses (Figure 3B). In contrast, the lipase immobilized with 0.8% (w/v) glutaraldehyde exhibited a loss of lipase activity with four uses.

## Conclusions

Optimal immobilization of the *C. rugosa* lipase on the ground biomass of *S. grossus* L.f. fibers was achieved by prior activation of the fibers with 2% (w/v) 3-APTES in distilled water followed by crosslinking with 0.2% (w/v) glutaraldehyde prior to immobilization of the *C. rugosa* lipase. A higher concentrations of glutaraldehyde is less efficient presumably because the unreacted aldehyde groups of glutaraldehyde remain after crosslinking with an amino group of the lipase and so can attach to other amino groups of the lipase, thereby reducing the lipase's activity.

In terms of FAEE biodiesel synthesis, the immobilized *C. rugosa* lipase-catalyzed hydrolysis-esterification of palm oil and bioethanol yielded a higher release of FFAs and conversion to FAEE than those of the free *C. rugosa*. With the 0.2% (w/v) glutaraldehyde immobilized *C. rugosa* lipase also exhibited moderate reuse ability for up to six reuses.



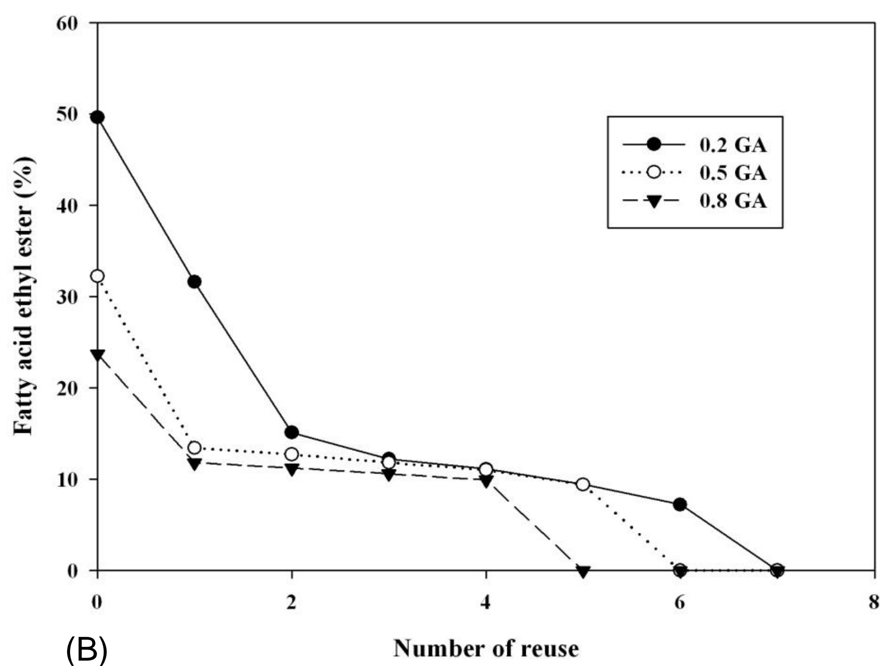




**Figure 3.** The % production of (A) free fatty acids (FFAs) and (B) fatty acid ethyl esters (FAEE; biodiesel) after repeated use of the immobilized *C. rugosa* lipase on *S. grossus* L.f. fibers crosslinked with various glutaraldehyde (GA) concentrations [as % (w/v)].

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# Impact of a blood-sucking parasite on the chemical composition of fatty acids in the white muscle of garfish (*Belone belone*, Belonidae) from Tunisian coasts (Central Mediterranean)

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**The objective of this study was to compare the composition of fatty acids in the garfish (*Belone belone*) white muscle removed from parasitized and non parasitized specimens. The chemical analysis results revealed low contents of total fatty acids for both parasitized and non-parasitized specimens. Their values, less than 2 g/100 g of fresh muscle, allowed the classification of the garfish as a lean fish. High ratios of saturated fatty acids were found in the garfish muscle reaching 58.4% of total fatty acids. These fatty acids were represented mainly by lauric, miristic and palmitic at a level of 50.3%. As a lean fish, garfish contains 16% polyunsaturated fatty acids (n-3). Two major fatty acids are docosahexaenoic and eicosapentaenoic with respective percentages of 9 and 1.17% of total fatty acids. The parasitized garfish showed increase in their fatty acids, mainly in pentadecanoic, pentadecenoic, docosahexaenoic and arachidonic acids and decreases in saturated acids especially lauric, miristic and palmitic. This drop is correlated with a very significant increase in PUFA from 16 to 26% of total fatty acids. In order to obtain 0.5 g/day of EPA + DHA, the amount of garfish required is 641 g of non-parasitized and 436 g parasitized fish.**

**Key words:** Garfish, blood-sucking, parasite, parasitized fish, fatty acid analysis, Tunisia.

## INTRODUCTION

Fish are known for their high content in protein and lipids. These, rich in polyunsaturated fatty acids (PUFA) n-3 such as eicosapentaenoic acid (EPA, 20: 5 n-3) and docosahexaenoic acid (DHA, 22: 6 n-3), are stored in various organs of the fish; liver, muscle, gonad, skin and perivisceral adipose tissue (Corraze and Kaushik, 1999). Specifically, it has been shown that the biochemical composition of muscle and PUFA content present significant differences according to its nature and its location in the various regions of the body; anterior, middle or caudal (Ben Smida et al., 2009). The quantitative variability of PUFA in a given organ is closely related to the life cycle of the fish. As such, Henderson and Tocher (1987) showed increased levels of muscle lipids in freshwater fishes according to the age and the size. Later, Mörköre and Rørvik (2001) confirmed these results in salmon. Jaloustre et al. (2012) found very large fluctuations in levels of triglycerides (TAG) in relation to

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**Abbreviations: PUFA,** Polyunsaturated fatty acids; **EPA,** eicosapentaenoic acid; **DHA,** docosahexaenoic acid; **TAG,** triglycerides; **GC,** gas chromatography; **TFA,** total fatty acids; **MUFA,** monounsaturated fatty acids; SFA, saturated fatty acid.

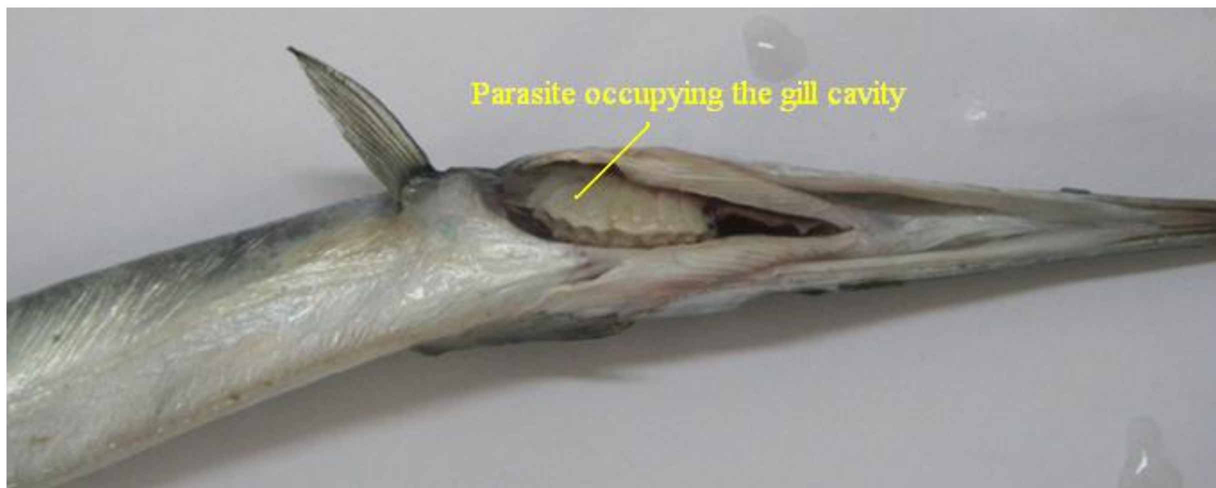


**Figure 1.** Photo showing a blood-sucking inside the garfish gill cavity (by Bedoui-Fehri R).

total lipids of muscle and liver of sardine and anchovy in relation to their sexual cycle; much more variable in the sardine (30-40% during the period of sexual rest against 5% in the period of the intense sexual activity). It is also important to mention that the fishes move in an open environment and live in constant dynamic interaction with their environment as adoption of diverse and complex behavioral strategies to insure their position in the food chain.

However, fish are never protected against the environmental stresses such as pollution and parasitism (Amilhat, 2007). Parasitism involves a wide diversity of species among the endo and ectoparasites of fish. However, the impact of ectoparasites on fish, including bloodsucking crustacean isopods remains little known and the available information is limited to the effect of the parasite on the outer aspect of the fish (Tombi et al., 2011). It is even reported that ectoparasites are not generally damaging to fishes and do not affect the quality of meat (Natural Resources Quebec, 2006-2013).

To our knowledge, no studies on the lipids biochemical composition of parasitized fish have been performed. It is in this context and in order to assess the real impact of the blood-sucking parasite on garfish from Tunisian coast, we undertake a comparative study of the fatty acid composition between parasitized and non parasitized fish.

## MATERIALS AND METHODS

Garfish samples examined were collected from fishermen in July 2011. This month corresponds to the spawning period of the fish (Bedoui et al., 2002). The total length and the weight of the non-infected specimens (n = 6) varied between 27 and 28.5 cm and 66.6 and 80.7 g, respectively. Those of infected specimens (n = 6) varied between 23 and 29.5 cm (length) and between 42.9 and 88.9 g (weight). The infection rate was one parasite by gill cavity (Figure 1).

In total, six parasites were collected. The parasite was identified as being an isopod belonging to *Mothocya belonae sp.* (Bruce, 1985). Approximately, a one-gram sample of white muscle was taken from the left anterior side of each specimen. The total lipid extraction was performed according to the method of Folch et al. (1957). The total lipids obtained were stored in chloroform - methanol- butylated hydroxytoluene (BHT) at -28°C. Before being analyzed by gas chromatography (GC), fatty acids were made volatile by esterification using sodium methylate (Cecchi et al., 1985). An internal standard, the nonadecanoate methyl $C_{19:0}$ (Sigma) were added in order to quantify the fatty acids. Methyl esters of total fatty acids were separated, identified, determined and titrated by a gas chromatograph type HP 6890 with a split/splitless injector with electronic pressure control and a flame ionisation detector. The apparatus was equipped with a capillary column HP Innowax 30 m long, with an internal diameter of 250 µm and whose film thickness was 0.25 µm. Polar stationary phase used in this column was polyethylene glycol. The oven temperature was programmed as follows: from 50 to 180°C at a rate of 4°C/min; from 180 to 220°C to 1.33°C/min; 220°C for 7 min. To obtain an accurate identification of the various fatty acids of the lipids of the garfish, we compared the retention times of the fatty acids in our samples with those of a mixture of methyl esters of reference (Supelco-3 PUFA). The results represent the average of six replicates (n = 6). For data analysis, each fish sample was subjected to a one-way analysis of variance, with a confidence level of 5%, using the Duncan test. Statistical analyzes were performed using the Statistical Package for the Social Sciences (SPSS) 13 software.

## RESULTS AND DISCUSSION

The content of muscle tissue (without the skin) of total fatty acids (TFA) of parasitized and non-parasitized garfish collected in July is shown in Table 1. These contents are less than 2 g/100 g (fresh muscle). According to the classification of Ackman (1994), garfish can be considered as a lean fish. This value (0.78 g) is close to that of garfish captured in May in the Baltic Sea, that was 2.44 g/100g (fresh muscle) (Kolakowska et al., 2000) and is low compared to that captured from October to March, in the Black Sea, 5 g/100 g (fresh muscle)

**Table 1.** Contents of TFA in the garfish white muscle (g/100g fresh muscle).

| Parameter | Garfish | | F | P |
|---|---|---|---|---|
| | Non parasitized | Parasitized | | |
| Total fatty acids) (g/100g fresh muscle) | 0.78 ± 0.13 | 0.68 ± 0.09 | 0.04 | * |

Mean ± ES; n=6; F: Fischer test; *Significant *P ≤ 0.05.*

(Boran and Karaçam, 2011). It seems that TFA from the garfish vary greatly depending on the season. This variation is related to seasonal environmental changes such as water temperature, the current patterns, the availability of prey resources whose impact on fish behavior are not negligible. We mention, as an example, periods of starvation or intense feeding activity, feeding or reproduction migration to other more suitable areas. Given that the garfish spawns intermittently, Kompowski (1965a, b) reported that this fish maintains its rate of feeding during the different stages of the sexual cycle. The long period of egg maturation reported by Zorica et al. (2010) could explain the low fat content in the non-parasitized fish (Table 1).

The TFA value increased from 0.78 g / 100 g (fresh muscle) in the non-parasitized fish to 0.68 g/100 g (fresh muscle) in parasitized fish (Table 1). On this subject, Macnab and Barber (2012) reported that infected fish behave differently; probably parasites affect their behavior, forcing them to move and look for warmer temperatures. This decrease in the amount of total fatty acids was confirmed by Van den Broek (1978) who found that the parasite *Lernaeocera branchialis*, caused, in its host, *Merlangius merlangus*, a significant decrease in body weight associated with a decrease in the lipid content of the liver and an increase in the rates of cholesterol. Thus, we suggest that the effect of parasitism would result in a decrease in the amount of total fatty acids in parasitized fish. The majority of fatty acids identified in the non-parasitized fish are lauric, the pentadécénoïque the palmitic and DHA with percentages of 25.6, 17.3, 14.8 and 9% of TFA. Saturated fatty acids are present with a percentage of 58.3% TFA. They are represented by the $C_{12:\,0}$, $C_{14:\,0}$ and $C_{16:\,0}$ that have a cumulative percentage of 50.3%. The biochemical composition of fish is strongly affected by the composition of their food (Orban et al., 2007). Hughes et al. (1996) have shown that stearic acid ($C_{18:\,0}$), palmitic ($C_{16:\,0}$) and myristic ($C_{14:\,0}$) or lauric acid ($C_{12:\,0}$), have different metabolisms and should be considered separately.

In all living organisms, monounsaturated fatty acids (MUFA) are provided from endogenous synthesis and from their alimentation (Legrand, 2007). According to Table 3, the pentadecenoic fatty acid ($C_{15:\,1}$) was identified as a major monounsaturated fatty acid. Oleic acid ($C_{18\,:1n\text{-}9}$), a characteristic of fish tissues (Steffens, 1997), was present at the percentages of 3.3 and 2.5% in the parasitized garfish muscle and non-parasitized garfish muscle, respectively. Kołakowska et al. (2000) observed in reared garfish percentages of 0.81% for $C_{15:\,1}$ and 24.2% for the $C_{18}$ complex: $_{1n\text{-}9}$, $C_{18:\,1n\text{-}7}$. We suggest that the $C_{15:\,1}$ is a product that does not come from endogenous synthesis, but rather comes from the garfish alimentation. The muscles of the garfish non-parasitized are poor in PUFA 16% TFA. This result is confirmed by the value of PUFA / saturated fatty acid (SFA) ratio which is 0.29 whereas the ratio recommended by the HMSO (1994) is 0.45. Among the fatty n-3 and n-6 acids, fatty acids DHA and arachidonic dominate with percentages of 9 and 1.9% TFA, respectively. Probably, the lack of fatty acid n-6 and n-7 might be attributed to environmental conditions and / or fish food. The ratio n-3/n-6 is a conclusive criterium to compare the nutritional value of fish oils (Piggot and Tucker, 1990).

Fish or fishery produce rich in fatty acids type n-3 fatty and poor in fatty acids type n-6 are beneficial to human health (Sargent, 1997). In the garfish, the ratio n-3/n-6 ratio is 5.9. The effect of the blood-sucking parasite on the muscle of the garfish results in a significant variation ($p \leq 0.01$) PUFA / SFA ratio, going from 0.29 to 0.63. This increase is explained, on the one hand, by a significant increase in the percentage of PUFA (16 to 26.4%) and, on the other hand, by a highly significant decrease in the percentage of the group, $C_{12:\,0} + C_{14:\,0} + C_{16:\,0}$, going from 50.3 to 9.7% TFA. The fatty acid profile of the parasitized garfish depends on the enzymatic activity of lipid metabolism vis-à-vis the SFA and MUFA. The parasitized muscles of garfish accumulate PUFAs with a transformation of SFA that is more important than that of MUFA. These results are corroborated by the ratio PUFA / MUFA and PUFA / SFA (Table 2) with respective values of 0.7 to 0.8 ($p > 0.05$) and 0.29 to 0.63 ($p \leq 0.01$).

At the level of PUFA, we note a significant increase in fatty acids of the type n-3 (13.1 to 20.9% TFA) and a very significant increase ($p \leq 0.001$) in the percentage of fatty acids type n-6 (from 2.2 to 5.1% TFA). The n-3 fatty acids are represented by the DHA with a percentage of 14.9% TFA, while those of type n-6 are represented by arachidonic with a percentage of 4.8% TFA acid. The value of the n-3/n-6 ratio is 4.2. Thus, the impact of the blood-sucking on the garfish shows a low value of this ratio. According to the study of Hossain (2011), a lower value of n-3/n-6 ratio means that the enzymes that convert fats into the forms in which they are active in the body are rather directed towards the synthesis of n-6 fatty acids. According to the study of Ackman (1980), fish are

**Table 2.** Composition on fatty acids in the garfish (*Belone belone*) from Tunisian coasts.

| Fatty acids | Garfish specimen | | P |
|---|---|---|---|
| | Non Parasitized | Parasitized | |
| C12 : 0 | 25.64±5.79[a] | 7.58±2.63[b] | * |
| C14 : 0 | 9.81±3.34[a] | 1.29±0.32[b] | * |
| C15 : 0 | 2.09±0.28[b] | 23.90±2.66[a] | * ** |
| C16 : 0 | 14.86±2.36[a] | 0.81±0.11[b] | ** |
| C17 : 0 | 1.03±0.10[a] | 1.30±0.10[a] | ns |
| C18 : 0 | 5.10±0.49[a] | 7.04±0.67[a] | ns |
| C15 : 1 | 17.37±6.04[a] | 23.60±2.56[a] | ns |
| C16 : 1n-9 | 2.21±0.31[a] | 1.27±0.11[b] | * |
| C16 : 1n-7 | 1.10±0.16[a] | 0.63±0.05[b] | * |
| C18 : 1n-9 | 2.45±0.37[b] | 3.27±0.43[a] | * |
| C18 : 1n-7 | 1.44±0.17[a] | 1.96±0.17[a] | ns |
| C20 : 1 | 0.35±0.12[a] | 0.24±0.07[a] | ns |
| C22 : 1 | 0.46±0.06[a] | 0.79±0.28[a] | ns |
| C16 : 2n-4 | 0.61±0.14[a] | 0.17±0.03[b] | * |
| C18 : 2n-6 | 0.19±0.07[a] | 0.29±0.13[a] | ns |
| C20 : 2n-6 | 0.14±0.00[a] | 0.07±0.01[b] | ** |
| C18 : 3n-3 | 0.04±0.01[b] | 0.11±0.03[a] | ** |
| C18 : 4n-3 | 1.20±0.64[a] | 0.27±0.18[a] | ns |
| C20 : 4n-6 | 1.90±0.20[b] | 4.79±0.37[a] | *** |
| C20 : 4n-3 | 0.25±0.04[a] | 0.44±0.11[a] | ns |
| C20 : 5n-3 | 1.17±0.05[b] | 2.26±0.20[a] | * |
| C22 : 5n-3 | 1.50±0.17[b] | 2.92±0.24[a] | * |
| C22 : 6n-3 | 9.00±0.88[b] | 14.89±1.65[a] | * |
| C12:0+C14 :0+C16 : 0 | 50.32±6.79[a] | 9.69±3.06[b] | ** |
| SFA | 58.36±6.68[a] | 41.74±2.16[a] | ns |
| MUFA | 25.41±5.80[a] | 31.80±2.21[a] | ns |
| PUFA | 16.02±1.08[b] | 26.24±1.22[a] | ** |
| UFA | 41.44±6.68[a] | 58.05±2.16[a] | ns |
| n-3 | 13.15±1.00[b] | 20.90±1.13[a] | ** |
| n-6 | 2.25±0.25[b] | 5.16±0.40[a] | *** |
| n-7 | 2.55±0.75[a] | 2.60±0.15[a] | ns |
| n-9 | 4.56±0.49[a] | 4.55±0.43[a] | ns |
| EPA+DHA | 10.17±0.84[b] | 17.16±1.47[a] | ** |
| n-3/n-6 | 5.95±0.49[a] | 4.21±0.38[b] | ** |
| UFA/SFA | 0.78±0.22[b] | 1.40±0.12[a] | * |
| PUFA/MUFA | 0.70±0.11[a] | 0.83±0.70[a] | ns |
| PUFA/SFA | 0.29±0.05[b] | 0.63±0.04[a] | ** |

SFA, $C_{12:0}$+$C_{14:0}$+$C_{15:0}$+$C_{16:0}$+ $C_{17:0}$+$C_{18:0}$; MUFA, $C_{15:1}$+ $C_{16:1n-9}$+ $C_{16:1n-7}$+ $C_{18:1n-9}$+ $C_{18:1n-7}$+ $C_{20:1}$+ $C_{22:1}$; PUFA, $C_{16:2n-4}$+ $C_{18:2n-6}$+ $C_{20:2n-6}$+ $C_{18:3n-3}$+ $C_{18:4n-3}$+ $C_{20:4n-6}$+ $C_{20:4n-3}$+ $C_{20:5n-3}$+ $C_{22:5n-3}$+ $C_{22:6n-3}$; UFA, MUFA+ PUFA; *, ** and ***, significant at P≤0.05, P≤0.01 and P≤0.001, respectively; ns, not significant (P>0.05). Mean ± ES; n=6; P<0.05.

inclined to adjust their lipid composition to the demands of the environment and to their own physiological requirements. Their behaviors and food preferences support this goal.

A number of countries such as Canada, United Kingdom, World Health Organization (WHO) and North Atlantic Treaty Organization have advocated dietary recommendations for PUFA (n-3). These recommendations are 0.3 to 0.5 g/jour EPA + DHA (Kris-Etherton et al., 2002). According to Table 3, these recommendations can easily be fulfilled through the consumption of garfish with a daily intake of 641 g if it is non-parasitized and 436 g if it is parasitized.

## Conclusion

It appears from this study that *M. belonae,* parasite of the

**Table 3.** Quantities of EPA+DHA in the garfish white muscle and proposed daily intake of fish flesh.

| Specimen | EPA+DHA (mg/100 g) | EPA+DHA (mg/150 g) | Quantity of consumed fish (g) to provide 0.5 g EPA+DHA/day |
|---|---|---|---|
| Non-parasitized | 78 | 117 | 641 |
| Parasitized | 115 | 172 | 436 |

garfish, modifies the fatty acid composition of its white muscle. This change improves the nutritional quality of this pelagic fish. Thus, it results in a significant decrease in the percentage of group $C_{12:0}$, $C_{14:0}$ and $C_{16:0}$, which rose from 50.3 to 9.7%. This effect is also confirmed by the increase in the UFA / SFA ratio by about 100% (0.78 to 1.40). The positive effect of this parasite allows a significant increase in PUFA, changing from 16 to 26.2% TFA. Because of both the low fat content of garfish and the need to guarantee the intake of 0.5 g EPA + DHA/day, the recommended intake of this fish is a portion of more than 150 g.

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*Full Length Research Paper*

# Novel antibacterial activity of *Terfizia claveryi* aqueous extract against clinical isolates of corneal ulcer

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***Terfizia claveryi* was examined for *in vitro* antibacterial activity using the disc diffusion, well diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). *T. claveryi* exhibited excellent antibacterial activity against all clinical isolates of corneal ulcer tested, especially against *Pseudomonas aeruginosa* which showed the maximum antibacterial activity with mean zone of inhibition 20.33 mm at concentration of 100 mg/ml. The MIC for *Staphylococcus aureus* ranged from 0.040-1.250 mg/ml and MBC for *Escherichia coli* was 75 µl/ml. In the present study, the MIC value of the active aqueous extract were lower than the MBC values suggesting that, *T. claveryi* aqueous extracts were bacteriostatic at lower concentration but bactericidal at higher concentration. Also, the bacterial zone of inhibition increased with the increasing concentration of *T. claveryi* aqueous extract. To the best of our knowledge, this is the first report for the novel antibacterial activity of *T. claveryi* aqueous extract. This active compound may be used as alternative therapeutic drug for the control of corneal infections. However, further research is needed to examine its *in vivo* mechanism of action, toxicity, and therapeutic effect.**

**Key words:** Bacteria, corneal infection, antimicrobials, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), *Terfizia claveryi.*

## INTRODUCTION

In the 21st century antibiotic resistance of clinical bacterial isolates are increasing drastically, the search for new and safe anti-bacterial compounds are important and natural medicinal products seems to be a logical and effective source for seeking new antimicrobial agents. In Saudi Arabi, the traditional medicinal practices have been known since ancient time for their unique properties and obvious therapeutic potential in treating a variety of diseases (Al-Bukhari and Al-Bukhari, 1996).

*Terfizia claveryi*, as brown desert truffles, are considered to be one of the oldest food stuffs known for their nutritional value especially when compared with meat and fish (Abu-Rabia, 1983). Truffle aqueous extract is used as a folk medicine in Gulf countries (Iraq, Saudi Arabia and Eastern Jordan) to treat eye infections (Bokhairy and Parvez, 1993). *T. claveryi* ascocarps contain 16% protein, 28% total carbohydrates, 4% total crude fiber, 2% total crude fat and rich in mineral as well as carbohydrate contents, with nine saturated and four unsaturated fatty acids and 29 amino acids, unique flavor, nutritional value and medicinal properties for a variety of ailments (Al-Delaimy, 1977). It is also used as a

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**Abbreviations: MIC,** Minimum inhibitory concentrations; **MBC,** minimal bactericidal concentration.

nourishing and invigorating preparation for convalescents in Mediterranean countries (Janakat et al., 2004). Corneal infection is one of the most common ocular diseases in both humans and animals and can lead to blindness (Olivier, 2003; Jatoi et al., 2002). There are various pathogenic organisms, like *Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus spp.* and *Staphylococcus epidermis*, reported to cause corneal infection (Wahl et al., 1991; Dart, 1988; Charteris et al., 1994; Leeming, 1999). Many antibacterial preparations are used to treat eye infections such as, chloramphenicol, fluoroquinolone, neomycin and aminoglycosides (Goldstein et al., 1999). However, the increasing resistance of many bacteria and the side effects to the currently used antibiotics are documented (Lancaster and Swart, 1998; Ostier, 1993; Vaughan and Asbury, 1980; Skies et al., 2007). The medicinally important *T. claveryi* fruit was selected in this study to investigate whether having antimicrobial activity against clinical isolates of corneal ulcer.

## MATERIALS AND METHODS

### Collection of *Terfizia claveryi*

"Kamma" the local name of *T. claveryi*, brown desert truffles was purchased in April-2012 from the local market in Riyadh, Kingdom of Saudi Arabia. The desert truffle fruit material identification was confirmed by Department of food and Agriculture, Qassim University, Kingdom of Saudi Arabia.

### Preparation of crude extract of *T. claveryi*

The collected *T. claveryi* fruits were cleaned and cut into small pieces and dried under shade at room temperature. The dried material was ground to fine powder using a mechanical blender and passed through 24 mesh sieve. *T. claveryi* powder (100 g) was extracted with 50 mM sodium phosphate buffer (pH 7.0) at 37°C. The extract was filtered through cheese cloth to remove the major debris and the filtrate was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was considered as crude aqueous extract of *T. claveryi* and stored at 4°C for experimental use.

### *In-vitro* antibacterial activity

### Test microorganisms

Microbial cultures of eight different strains of both Gram positive and Gram negative bacteria were used for the determination of antibacterial activity. Gram-positive *(S. aureus, S. epidermidis, Staphylococcus faecalis)* and Gram-negative *(Escherichia coli, P. aeruginosa, Pseudomonas vulgaris, Klebsiella pneumonia),* clinical bacterial isolates were used. All the bacterial strains were sub-cultured at 37°C on Mueller-Hinton agar (Oxoid, Hampshire, UK) slants every 15 days and stored at 4°C. The bacterial isolates were obtained during parallel studies from clinical cases that suffered corneal infections and subjected to several hospitals at Qasssim region during 2012. Sampling, culturing, isolation and identification were done in the Department medical laboratory at College of Applied Medical Sciences, Qassim University using the standard Microbiology techniques (Collee et al., 1996).

### Antibiotic susceptibility testing

The microorganisms were tested for their sensitivity against the antibiotics including: Ciprofloxacin (5 µg), tetracycline (30 µg), gentamycin (10 µg), tobramycin (10 µg), erythromycin (15 µg), moxifloxacin (5 µg), cefoxitin (30 µg), oxacillin (1 µg), clotrimazole (10 µg). The susceptibilities of the isolated pathogens were determined by the modified Kirby-Bauer disc diffusion method with Muller Hinton agar plates (Bauer et al., 1996). All the media used in the present investigation were obtained from Oxoid, Hampshire, UK.

### Agar well diffusion method

Antibacterial activity of *T. claveryi* was determined by agar well diffusion method (Bauer et al., 1996). One hundred microliter (100 µl) of standardized inoculum (0.5 Mac-Farland) of each test bacterium were inoculated on molten Mueller-Hinton agar, homogenized and poured into sterile plates. Standard cork borer of various diameter (6, 16 and 20 mm) were used to make uniform wells into which different amounts of aqueous extract of *T. claveryi* (100, 300 and 500 µl) were added. Standard antibiotic ciprofloxacin was used as positive control and 50 mM sodium phosphate buffer alone was used as negative control. The plates were then incubated at 37 ± 1°C for 24 h. The experiments were carried out in triplicates and the zone of inhibition was measured with the help of standard scale.

### Determination of minimum inhibitory concentrations (MIC)

The MIC of *T. claveryi* was determined by macro dilution method (Weckesser et al., 2007). Several dilutions of *Terfizia* aqueous extracts ranges (0.040-1.250 mg/ml) and standard antibiotic ranges (0.024-0.240 mg/ml) were prepared from stock solutions by serial dilution technique. Each sample dilution were mixed properly with 20 ml of sterile molten Muller Hinton agar and poured into 90 mm plates and allowed to cool under laminar air flow before streaking with 10 µl of 0.5 McFarland standards. The lowest concentration which did not show any macroscopic growth of tested microorganism was identified as the MIC.

### Determination of minimal bactericidal concentration (MBC)

The MBC of the *T. claveryi* extracts were determined by a macro broth dilution method (Perez et al., 1990). Each set contains 8 tubes as follows: Positive control, negative control, positive control with 50 mM potassium phosphate buffer pH 7 where 50, 75, 100, 125, 150, 175 and 200 µl of *T. claveryi* aqueous extract were added for each bacterial species. 7 tubes out of eight were inoculated with 10 µl of single bacterial species. The plates were then incubated at 37°C overnight and the lowest dilution that yielded complete inhibition of bacterial growth was taken as the MBC. Each of the extract was tested in triplicate and the average values were obtained for two repeated experiments.

### Statistical analysis

These parameters were tested in triplicates. The values were expressed as mean ± standard deviation (SD), mean value ± standard error of the mean (SEM) of growth inhibition zones diameters obtained with aqueous extract which amount was sufficient to perform repetitions. Statistical differences between the two variants of diffusion method were detected by analysis of variance (ANOVA) followed by Duncan test, the statistical analysis was performed using SPSS statistical software.

**Table 1.** Mean zone of inhibition (mm) of *T. claveryi* aqueous extracts against bacterial isolates in comparison with standard antibiotic.

| Bacterial isolate | Mean zone of inhibition (mm) *(mean ± SD)* | |
|---|---|---|
| | ***T. claveryi*** **(100 mg/ml)** | **Standard antibiotic (ciprofloxacin 5 mg/disc)** |
| *Staphylococcus aureus* | 19.00± 1.00 | 27.33±0.57 |
| *Staphylococcus epidermidis* | 18.00± 1.00 | 29.00±0.57 |
| *Streptococcus faecalis* | 17.00± 1.00 | 28.66± 1.52 |
| *Escherichia coli* | 15.33± 0.57 | 30.33± 1.52 |
| *Pseudomonas aeruginosa* | 20.33± 1.00. | 26.00± 1.52 |
| *Proteus vulgaris* | 15.33± 0.57 | 27.66± 0.57 |
| *Klebsiella pneumonia* | 14.66± 0.57 | 28.33± 1.52 |

## RESULTS

### Mean zone of inhibition

In the present study, all the tested bacteria were sensitive to *T. claveryi* aqueous extract. Among the Gram positive and Gram negative; S. *aureus, P. aeruginosa* exhibited highest rate of sensitivity to *T. claveryi* aqueous extract. This study also reveals that the *P. aeruginosa* was highest susceptible bacteria with 20.33 mm zone of inhibition followed by *S. aureus* (19.00 mm), *S. epidermidis* (18.00 mm), *S. faecalis* (17.00 mm), *E. coli* (15.33 mm), *P. vulgaris* (15.33 mm) and *K. pneumonia* (14.66 mm) at the test concentration of 100 mg/ml, which was comparable to standard antibiotic ciprofloxacin 5 mg/disc (Table 1).

### Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Our results exhibited the broad spectra of antibacterial activity of *T. claveryi* aqueous extract (Figure 1). The MIC and MBC of *T. claveryi* aqueous extract against clinical isolates has been listed in (Tables 2 and 3). The MIC ranged between 0.40 to 1.25 mg/ml and the MIC of ciprofloxacin ranged between 0.024 to 0.240 mg/ml. Among the Gram positive bacteria strain, *S. aureus* isolates were found to be more sensitive than Gram negative *K. pneumonia* strains. *T. claveryi* extracts exhibited the greatest antibacterial activities as determined by the MBC. The MBC ranged between 75 to 120 µl/ml.

The lowest MBC value was observed towards *S. aureus* (75 µl/ml) and *K. pneumonia (*85 µl/ml). The inhibition of tested bacteria was increased by the increase in the amount of the *T. claveryi* aqueous extract (Table 4 and Figure 2).

In all clinical bacterial isolates, antibiotic susceptibility to eight antibiotics was accessed by Kirby-Bauer disc diffusion method (Table 5). All bacterial isolates were sensitive to ciprofloxacin while *S. aureus* and *S. faecalis* were resistant to erythromycin.

## DISCUSSION

Saudi Arabia has old history in the use of diverse herbal medicines for traditional healing. Literature overview shows that, the most common species of the genus Terfizia especially *Terfizia claveryi* are round, tan to brown like small sandy potatoes, have unique flavor, nutritional value and medicinal properties. It is used for treatment of variety of ailments like eye infections, open cuts, stomach ailment among others (Goldstein et al., 1999; Lancaster and Swart, 1998). In Gulf countries, Truffle is used in the form of flour or juice for curing various infections in folk medicine.

Numerous studies have been reported in the past, focusing on antimicrobial activity of aqueous extract of *Asparagus racemosus1*, *Asphodelust enuifolius, Balanites aegyptiaca, Eclipta alba, Pedalium murex, Ricinuscommunis, Trigonellafoenumgraecum, TrianthemadecandraL, Argemone mexicana, Tinosporacordifolia* and *Cassia fistula* against various bacteria and fungi by using well diffusion and disc diffusion methods (Satavat and Gupta, 1987; Kaul (1997); Valsaraj et al., 1997; Khafagy and Ishrak, 1999; Mandal et al., 2000; Geethalakshmi et al., 2010; Rahman et al., 2011; Upadhyay et al., 2011). The antimicrobial analysis using the agar well diffusion method and MIC value had been used by many researchers (Arora and Kaur, 2007; Gurudeeban et al., 2010; Pavithra et al., 2010). However, since no studies have been reported on the antimicrobial activity of *T. claveryi* extracts against bacterial isolates causing corneal ulcer, we made this attempt.

Several herbal medicines like *Pothomorphe umbellate* extract and Chinese herbal medicine (emodin) have been reported to play an important role in the therapeutic activities of corneal ulcerative problems (Barros et al., 2007; Kitano et al., 2007). The effects of *S. jalambrensis* preparation on ocular inflammation have been explored by *in vivo* topical administration. The scientific research on the antimicrobial potency of many of the plants and herbs used for medicinal purposes in Saudi Arabia is lacking.

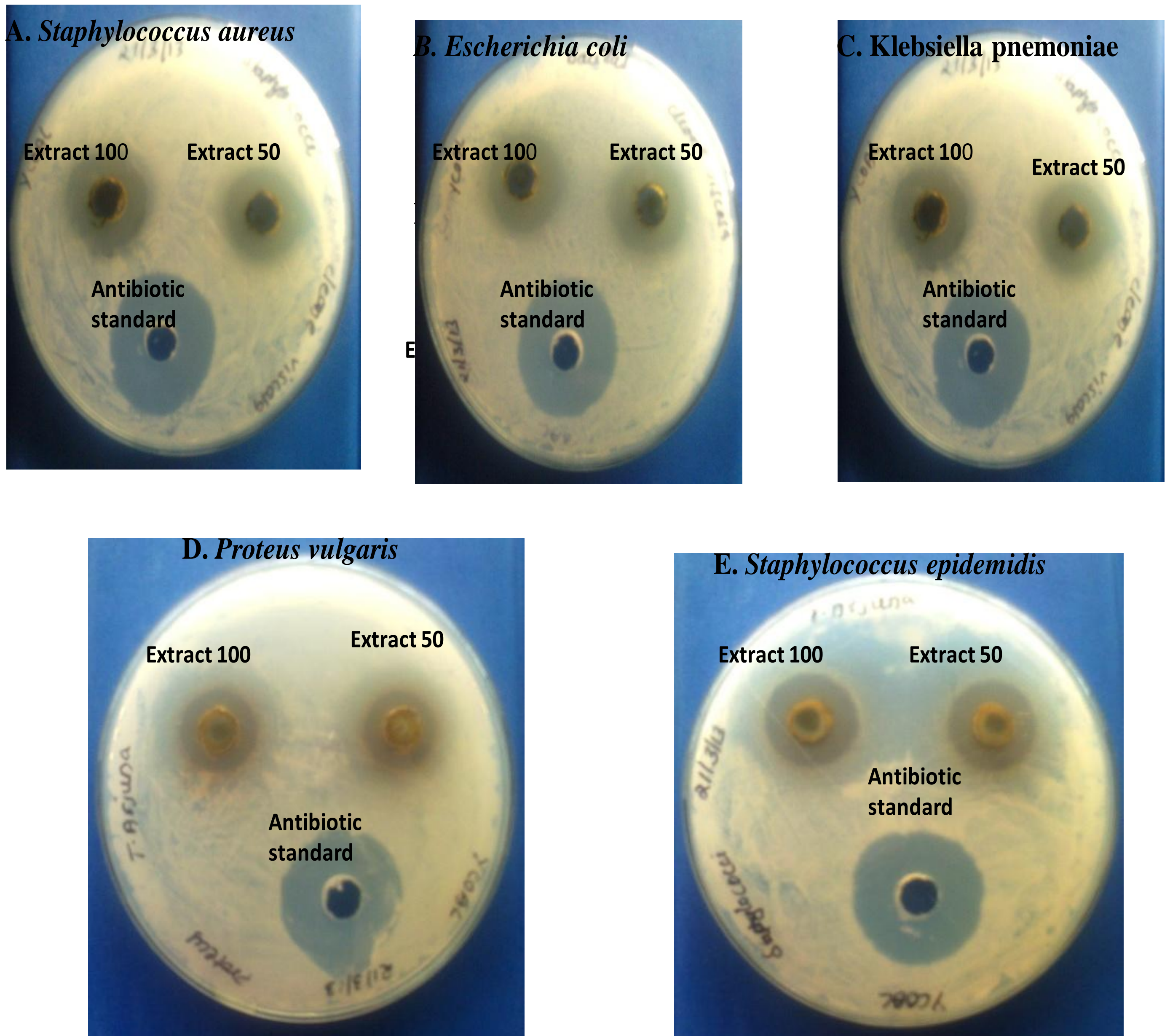


**Figure 1.** Efficiency of *Terfizia claveryi* aqueous extract against different bacteria compared with standard antibiotic (ciprofloxacin).

**Table 2.** Minimum inhibitory concentrations (MICs) of *T. claveryi* aqueous extract against bacterial isolates in comparison with standard antibiotic.

| Bacterial isolate | MIC value (mg/ml) | |
|---|---|---|
| | *T. claveryi* | Standard antibiotic (ciprofloxacin) |
| *Staphylococcus aureus* | 0.40 | 0.024 |
| *Staphylococcus epidermidis* | 0.40 | 0.024 |
| *Streptococcus faecalis* | 0.75 | 0.120 |
| *Escherichia coli* | 0.60 | 0.195 |
| *Pseudomonas aeruginosa* | 0.55 | 0.240 |
| *Proteus vulgaris* | 1.20 | 0.124 |
| *Klebsiella pneumonia* | 1.25 | 0.110 |

**Table 3.** Minimum Bactericidal concentrations (MBC) of *T. claveryi*a aqueous extract against bacterial isolates in comparison with standard antibiotic.

| Bacterial isolate | MBC value (µl/ml) | |
|---|---|---|
| | *T. claveryi* | Standard antibiotic (ciprofloxacin) |
| *Staphylococcus aureus* | 75 | 0.024 |
| *Staphylococcus epidermidis* | 75 | 0.024 |
| *Streptococcus faecalis* | 90 | 0.120 |
| *Escherichia coli* | 95 | 0.195 |
| *Pseudomonas aeruginosa* | 100 | 0.240 |
| *Proteus vulgaris* | 120 | 0.124 |
| *Klebsiella pneumonia* | 85 | 0.110 |

**Table 4.** Inhibition zone diameter by *T. claveryi* aqueous extracts against tested bacterial isolates.

| Test organism | *T. claveryi* aqueous extract | | |
|---|---|---|---|
| | 100 µl | 300 µl | 500 µl |
| | Well (6 mm) | Well (16 mm) | Well (20 mm) |
| *Staphylococcus aureus* | 19 | 34 | 41 |
| *Staphylococcus epidermidis* | 20 | 33 | 40 |
| *Streptococcus faecalis* | 17 | 27 | 32 |
| *Escherichia coli* | 18 | 28 | 33 |
| *Pseudomonas aeruginosa* | 16 | 26 | 30 |
| *Klebsiella pneumonia* | 16 | 26 | 30 |
| *Proteus mirabilis* | 20 | 32 | 36 |

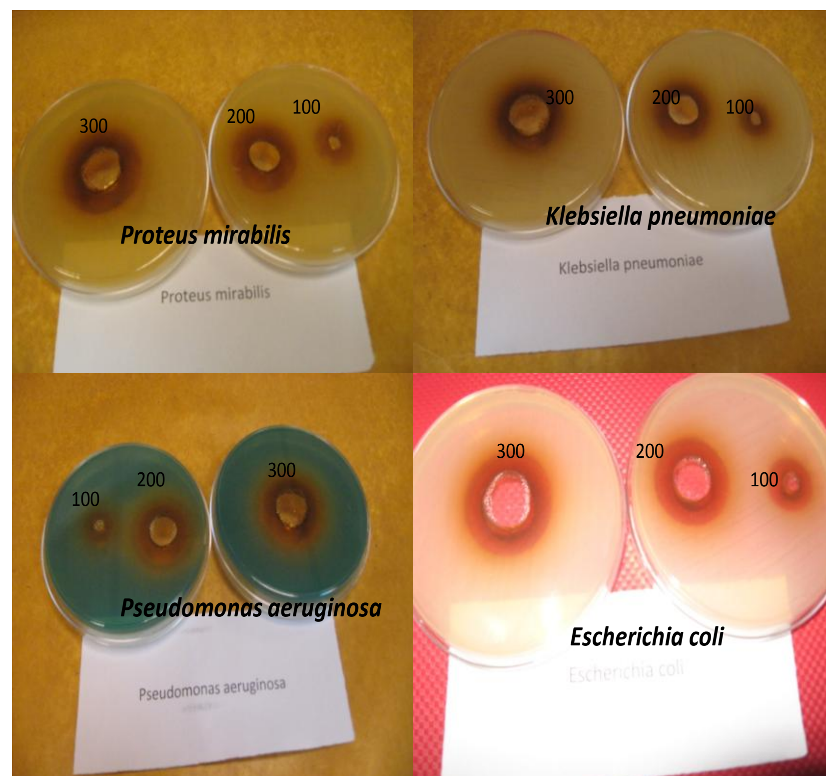


**Figure 2.** Effect of *Terfizia claveryi* aqueous extract against tested bacteria isolates.

**Table 5.** Antibiotic susceptibility pattern of Gram positive and Gram negative bacteria.

| Test organism | CIP | CN | TE | TOB | E | MXF | FOX | OX |
|---|---|---|---|---|---|---|---|---|
| *Staphylococcus aureus* | S | S | S | S | R | S | R | R |
| *Staphylococcus epidermidis* | S | S | S | S | S | S | S | S |
| *Streptococcus faecalis* | S | R | R | S | R | S | R | ND |
| *Escherichia coli* | S | S | S | S | S | S | S | ND |
| *Pseudomonas aeruginosa* | S | S | R | S | S | S | R | ND |
| *Klebsiella pneumonia* | S | S | S | S | S | S | S | ND |
| *Proteus mirabilis* | S | R | R | S | R | S | S | ND |

ND, Not determined; R, resistance; S, sensitive; CIP, ciprofloxacin; CN, cefoxitin; TE, tetracyclin; TOB, tobramycin; MYX, moxifloxacin; FOX, gentamycin; OX, oxicillin.

So, the present investigation evaluates the *in-vitro* antimicrobial activity of crude extracts of *T. claveryi* against the clinical bacterial isolates causing corneal ulcer. In the present study, we found that, *T. claveryi* extracts showed good antibacterial activity against most of the clinical bacterial isolates from the corneal ulcer cases (Table 1 and Figure 1).

The results of the current study clearly demonstrate that, aqueous extracts of *T. claveryi* could inhibit the growth of several bacterial pathogens causing cornea ulcer. However, the effectiveness varied against the different tested bacterial isolates. We determined the potential antibacterial activity of *T. claveryi* aqueous extract against seven clinical bacterial isolates causing corneal ulcer. The zone of inhibition for the *T. claveryi* extracts obtained by disc diffusion were equal or larger than those of the eight antibiotics commonly used to treat corneal ulcer infections (Table. 5).

In the present study, the MIC value of the active aqueous extract were lower than the MBC values suggesting that, the *T. claveryi* aqueous extracts were bacteriostatic at lower concentration but bactericidal at higher concentration (Tables 2 and 3). The mean zone of inhibition as shown in (Table 4 and Figure 2) was increased by the increase in the amount of the *T. claveryi* aqueous extract (Maji et al., 2010). The current results highlights, the fact that *T. claveryi* aqueous extracts exhibited antimicrobial activity against Gram positive and Gram negative bacteria isolated from corneal ulcer. Considering that these *T. claveryi* are edible and are traditionally used for treatment of a number of ailments, their anti-bacterial activity is quite significant and could present alternative treatments for corneal ulcer.

## Conclusion

To the best of our knowledge, this is the first report for the novel anti-bacterial activity of *T. claveryi* aqueous extract against bacterial isolates causing corneal ulcer. This active compound may be used as an alternative therapeutic drug for the control of corneal infections. Further research is needed to examine its *in-vivo* mechanism of action, toxicity, and therapeutic effect.

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## *UPCOMING CONFERENCES*

### 2nd Conference of Cereal Biotechnology and Breeding (CBB2), Budapest, Hungary, 5 Nov 2013

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