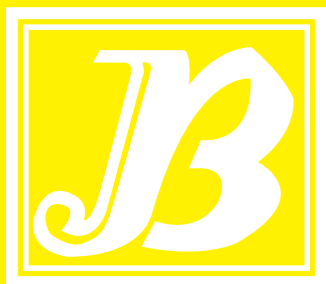


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MICROBIOLOGICAL QUALITY ASSESSMENT OF RAW SALAD VEGETABLE SOLD IN MINNA METROPOLIS, NIGERIA

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Abstract

Vegetables are edible part of plants. A total of twenty five raw salad vegetables were collected and the microbiological assessment was made using pour plate method. The analysis was carried out on carrots, cucumber, cabbage, lettuce and tomatoes. The results obtained from this study revealed that the total heterotrophic viable bacterial counts, coliform counts and fungal counts for all the salad vegetables ranged from 1.4×10^6 - 6.2×10^6 cfu/g, 1.1×10^6 - 3.3×10^6 cfu/g and 2.1×10^3 - 4.5×10^5 cfu/g respectively. The data were subjected to One Way Analysis of Variance (ANOVA) test which showed that there was significant difference ($p < 0.05$) in the microbial load of each of the raw salad vegetables samples. The microbial isolates identified were *E. coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella* sp., *Pseudomonas* sp., *Aspergillus niger*, *Mucor* sp., *Penicillium* sp., *Aspergillus flavus* and *Fusarium* sp. *Staphylococcus aureus* and *Aspergillus niger* were predominant. This suggests that salad vegetables used in this study are of public health concern because, they harbour microorganisms that could be hazardous to human health. Hence consumers should practice appropriate hygiene during the preparation of salad for consumption.

Key words: Assessment, microorganisms, quality, raw salad, vegetables

Introduction

Vegetables are considered as the leafy outgrowth of plants or plants shoot used as food. These include those plants or plant part used in making soup or served as an integral part of main meal (Yusuf et al. 2004). Vegetables can also be regarded as the edible component of plants, such components includes leaves, stalk, roots, tubers, bulbs, flowers and seed (ICMSF- International Commission on Microbiological Specification for Foods, 1974). Vegetables are important protective food and highly beneficial for the maintenance of health and prevention of diseases. They contain valuable food ingredients which are essential for the proper function of the body. Vegetable contain various medicinal and therapeutic agent and are valued mainly for their high vitamin and mineral content (Yusuf et al. 2004). Studies have evaluated the association of vegetables consumption with the reduction of risk of specific diseases (Hung et al. 2004). The occurrence of microorganisms in vegetables may be expected to reflect the sanitary quality of the processing steps and the microbiological condition of the raw product at the time of processing. Vegetables contamination in the field has been recognized as a source of human infection. Many of the viruses, bacteria and protozoan on vegetables which have caused food poisoning are derived from human feces (Ho et al. 1989, Rosenblum et al. 1990).

Foods, microorganisms and humans are in a long and interesting association that developed long before the beginning of recorded history. Foods are not only of nutritional value to those who consume them but often are ideal culture media for microbial growth (Willey et al. 2008). Vegetable which is defined as "any of the various plants especially herbaceous plants used wholly or partly for food" (Thomas 1995). Some of these

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vegetables are cooked while others are eaten raw such as carrots, cabbage and lettuce. Some of these vegetables are grown with application of manure, fertilizer, and irrigation water which may serve as possible sources for contamination of the vegetables (Buck et al. 2003). Minimally processed ready-to-eat vegetables consist of raw vegetables that have been washed, peeled, sliced, chopped and shredded. Salads may be served with or without dressings depending on the consumer. For many types of raw or minimally processed foods, the microbial load is often composed of mixed species (Schuenzel and Harrison 2002). The washing process of vegetables reduces the microbial load approximately 10 folds thereby prolonging the shelf life (Gracia-Gimeno and Zurera-Cosano 1997). The survival and growth of pathogens on fresh product like vegetables is influenced by the organism, the product and the environmental conditions in the field and there after including storage conditions (Birbeck 2004). Environmental conditions can greatly influence microbial population due to the presence of free moisture on leaves from precipitation, dew or irrigation which may promote survival and growth of microbial pathogens (Brock and Lindow 1999). During vegetable handling, the process of washing and shredding can transfer microorganisms. Pathogenic microorganisms are able to infiltrate cracks, crevices and intracellular spaces of food products.

However, pathogenic microorganism of human origin may also be present in minimally processed vegetables as the minimal technological processing may be unable to remove the original contamination resulting from air, soil, water, insects, animals, workers, harvesting and transportation equipment. Certain fungi such as *Aspergillus*, *Fusarium*, and *Penicillium* sp. as commonly occurring filamentous fungi grow in vegetable and their growth may result in production of toxins known as mycotoxins, which can cause a variety of ill effect in human from allergic responses to immunosuppression and cancer (Pitt et al. 1998).

Microbial growth on raw vegetables can result in the formation of biofilms by spoilage microorganisms which could provide protective environment for pathogens and reduce the effectiveness of sanitizers and other inhibitory agents. For example *Listeria monocytogene* in a multi species biofilm with *Staphylococcus aureus* has been reported to be essentially unaffected by treatment with 500 ppm chlorine (Ketchum 2002). Biofilms have been observed on numerous leaf surfaces including leaves of lettuce and cabbage (Morrisa and Monier 1997). Fresh cut vegetables are considered a potential hazard since the occurrence of pathogens cannot be excluded and the product is consumed without heating. Thus, ready-to-eat salad vegetables could be a potential pathogen source if not hygienically processed before consumption.

So far to the best of our knowledge, efforts have been geared towards studying the nutritional constituents of salad vegetables. As such there seem to be dearth of information on the microbiota been documented proving that a well-developed understanding of these is needed. Therefore, this study represents one of the few studies in this area. This will provide an insight to the microbiological characteristics of salad vegetables so as to lay a foundation of the microbiological aspects of salad vegetables in order to enhance better understanding of the microorganisms associated with salad vegetables particularly some pathogenic microbes that could cause health hazard and human diseases. Thus the study was designed particularly to determine the quality and microorganisms associated with salad vegetables in Minna, Niger state, Nigeria.

Materials and Methods

Collection of samples

A total of twenty five different raw salad vegetables were obtained from five different vendors. The samples included cabbage, tomatoes, cucumber, lettuce, and carrots (5 each) which were all obtained from different road side vendors within Minna, metropolis, Nigeria. All the samples were collected in sterile plastic bags and transported to the laboratory for processing immediately after collection.

Methodology and preparation of samples

The pour plate method was used as described by Jolt (2003) where 1ml of the appropriate serially diluted sample was transferred into labeled, well cleaned sterile Petri dish and molten agar medium (20 ml) poured. Poured plates were swirled gently to allow for proper distribution of colonies.

Bacteriological analysis

Total heterotrophic viable bacterial count

The method of Jolt (2003) was used where a 10 fold serial dilution of the samples was carried out. One gram of each of the salad vegetable was aseptically weighed and added into a sterile test tubes containing 9 ml of sterile, distilled water (deionized water). The test tubes were well shaken to mix together the content. Serial dilution was carried out using the mixed sterile distilled water as diluents. The 10-fold serial dilution was made by aseptically transferring 1 ml of the mixed distilled water in the test tubes into sterile test tubes containing 9 ml of sterile, distilled water. This gave ten times dilution. Subsequent dilutions were made from the aforementioned dilution. About 1 ml of the sample was pipette out from the 10^{-6} and 10^{-8} dilution tube into well labeled petri dishes. Then 20 ml of the molten nutrient agar was added into each plate and swirled gently to allow for proper mixing. The plates were incubated for 24 h at 37°C. Then the colonies develop on the plates were counted using a colony counter and expressed as colony forming unit per gram (cfu/g). The sample from each vendor was examined in triplicates and the average was recorded. The colonies differing in size, shape and colour were selected from the different plates on nutrient agar and sub-cultured repeatedly to obtain pure isolates. Macconkey agar was used to determine coliform counts. The pure isolates were maintained on agar slant for further characterization and identification.

Mycological analysis

The fungal count was carried out by pipetting 1 ml of the serially diluted salad vegetable onto Sabouraud Dextrose Agar (SDA) containing 0.01% chloramphenicol. An appropriate dilution of 10^3 was used using pour plate method as described by Jolt (2003). The plates were incubated for 3 days at 25°C.

Characterization and identification of isolates

Bacterial isolates

The characterization and identification of the bacterial isolates were carried out based on cell morphology, Gram's reaction and biochemical tests according to methods described by Jolt (2003) and Oyeleke and Manga (2008). The isolates were identified by comparing with those of known taxa using the schemes of Cao (1996).

Fungal isolates

The mould isolates were characterized based on the colour of aerial and substrate hyphae, type of hyphae, shape and kind of asexual spores, presence of foot cell, sporangiophore, conidiophores, and the characteristics of spore head. A small portion of the mycelia growth was carefully picked with the aid of a sterile inoculating needle and placed in a drop of lactophenol cotton blue on a microscopic slide and covered with a cover slip. The slide was examined under the microscope, first with ($\times 10$) and then with ($\times 40$) objective lens to detect the spores and some special structures of the fungi. The isolates were identified by comparing their characteristics with those of known taxa using the schemes of Domsch and Gams (1970).

Results

Microbial counts

The results obtained for total viable bacterial counts, total coliform counts and total fungi counts for all the salad vegetables obtained in the present study ranged from 1.4×10^6 cfu/g - 6.2×10^6 cfu/g, 1.1×10^6 cfu/g - 3.3×10^6 cfu/g and 2.1×10^3 cfu/g- 4.5×10^5 cfu/g respectively (Table 1).

Frequency of occurrence of microorganisms isolated from salad vegetables

Table 2 revealed the frequency of occurrence of microbial isolates. *Staphylococcus aureus* and *Aspergillus niger* had the highest frequency of occurrence of 24.0 and 18.5 respectively.

Table 1. Total Microbial counts of the isolates.

Samples	Microbial counts (cfu/g)		
	Total coliform	Total viable	Total fungi
Cucumber	$1.3 \times 10^{6ab} - 3.3 \times 10^{6a}$	$2.3 \times 10^{6bc} - 6.2 \times 10^{6a}$	$2.1 \times 10^{3c} - 6.3 \times 10^{4c}$
Carrot	$1.1 \times 10^{6c} - 2.2 \times 10^{6a}$	$2.8 \times 10^{6c} - 3.0 \times 10^{6c}$	$1.8 \times 10^{5b} - 4.5 \times 10^{5a}$
Cabbage	$1.2 \times 10^{6bc} - 3.2 \times 10^{6a}$	$1.4 \times 10^{6c} - 2.1 \times 10^{6c}$	$1.9 \times 10^{4c} - 1.2 \times 10^{5b}$
Lettuce	$2.1 \times 10^{6a} - 2.2 \times 10^{6a}$	$1.4 \times 10^{6d} - 2.8 \times 10^{6a}$	$5.0 \times 10^{4c} - 1.2 \times 10^{5b}$
Tomato	$2.0 \times 10^{6ab} - 3.1 \times 10^{6a}$	$1.8 \times 10^{6bc} - 2.0 \times 10^{6c}$	$2.1 \times 10^{3d} - 1.2 \times 10^{4c}$

Values on the same column with different superscript (a, b, c, d) are significantly different from each other ($p < 0.05$) while those with the same superscript (a, b, c, d) are not significantly different from each other ($p > 0.05$).

Table 2. Frequency of occurrence of microorganisms isolated from salad vegetables.

Organisms	No of occurrence	Frequency of occurrence (%)
<i>Klebsiella</i> sp.	04	7.40
<i>Bacillus subtilis</i>	08	14.8
<i>Staphylococcus aureus</i>	13	24.0
<i>Escherichia coli</i>	05	9.30
<i>Aspergillus niger</i>	10	18.5
<i>Mucor</i> sp.	04	7.40
<i>Aspergillus flavus</i>	03	5.60
<i>Pseudomonas</i> sp.	02	3.70
<i>Fusarium</i> sp.	03	5.60
<i>Penicillium</i> sp.	02	3.70
Total	54	100%

Discussion

The results of the present study revealed that there was remarkable bacteria and fungi contamination of different salad vegetable samples. The samples were contaminated with varying degrees of pathogenic bacteria and fungi. The bacterial isolates identified in the present study include; *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas* sp, *Bacillus subtilis*, and *Klebsiella* sp. The bacterial count in general, ranged from 1.1×10^6 cfu/g - 6.2×10^6 cfu/g and the fungal count ranged from 2.1×10^3 cfu/g - 4.5×10^5 cfu/g. This is in agreement with the report of Adebayo et al. (2012) who also reported similar microbial counts. The high count of bacteria detected in salad vegetables in the present study may be due to improper handling during harvesting and transportation. In the present study, all the vegetables examined harbored *Staphylococcus aureus*, while other microorganisms include *Bacillus subtilis*, *Klebsiella* sp., *E. coli* and *Pseudomonas* sp.

Most strains of *Staphylococcus aureus* are known to be pathogenic due to the heat stable enterotoxin they produce. The presence of *Staphylococcus aureus*, a pathogenic organism of public health concern and significance in these vegetables might have contaminated the vegetables from source as a result of handling by farmers or retailers. During processing, contamination could arise by use of dirty hands or clothing by food handlers and utensils used in slicing or keeping the vegetables. Improper handling and improper hygiene might lead to the contamination of food and this might eventually affects the health of the consumers (Omemu and Bankole 2005, Mgbakor et al. 2011).

The detection of *E. coli* in the study revealed poor hygienic standard in the handling of these raw salad vegetables or it could also be from contamination during harvest, it can also be present in water using in washing the salad vegetables. Presence of *E. coli* indicates recent contamination by faecal matter and possible presence of other enteric pathogens known to be causative agents of food borne gastroenteritis and bacterial diarrhea disease (Adebayo et al. 2012).

The fungal isolates obtained in the present study include, *Aspergillus niger*, *Aspergillus flavus*, *Fusarium* sp, *Mucor* sp and *Penicillium* sp. The presence of these fungi in raw salad vegetables have been reported in previous studies (Akintobi et al. 2011, Al-Hindi et al. 2011). Generally, fungi are considered toxigenic or pathogenic (Al-Hindi et al. 2011). During refrigeration, some moulds may produce mycotoxins on raw salad vegetables (Al-Hindi et al. 2011). Pathogenic fungi, on the other hand, could cause infections or allergies (Monso 2004, Al-Hindi et al. 2011). *Aspergillus* sp are known to produce several toxic metabolites, such as aflatoxin, malformins, naphthopyrones and they can produce ochratoxins (OTA), a mycotoxin which is a very important toxin worldwide because of the hazard it poses to human and animal health thus extra care should be taken during personnel handling of these vegetable, harvesting, cleaning, sorting, packaging, transport and storage (Pitt et al. 1998, Petzinger and Weidenbach 2002, Al-Hindi et al. 2011). The result of the present study shows that the salad vegetables were contaminated by potentially pathogenic microbes. Therefore, salad vegetables could be a source of infection to the consumers if not properly washed before consumption (Al-Hindi et al. 2011).

Conclusion

The results of the present study revealed that the salad vegetables were all contaminated by a wide variety of potential pathogenic microorganisms. Proper hand wash with warm water and soap before handling the vegetable should be encourage. The vegetable should be thoroughly washed with clean water during the process of preparation. Food handlers should be advised on the need for good hygiene and the use of potable water for the washing of these vegetables to reduce the microbial load as low as possible.

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A COMPARATIVE STUDY OF BIOMARKER GENE SELECTION METHODS IN PRESENCE OF OUTLIERS

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Abstract

The main purpose of gene expression data analysis is to identify the biomarker genes by comparing the gene expression levels between two different groups or conditions. There are several methods to select biomarker genes and many comparative studies have been performed to select the appropriate method. However, they did not consider the problems of outliers in their data sets though it is very essential to select the method from robustness point of view due to outliers may occur in the different steps of the gene expression data generating process. In this paper, it is evaluated the performance among five popular statistical biomarker gene selection methods viz. T-test, SAM, LIMMA, KW and FCROS using both simulated and real gene expression data sets in absence and presence of outliers. In the simulated data analysis, it was demonstrated the performance of these methods in terms of different performance measures such as TPR, TNR, FPR, FNR and AUC and based on these measures, it was found that in absence of outliers, for both small-and-large sample cases all the methods perform almost similar. Whereas, in presence of outliers, for small-sample case only the FCROS method perform well than other methods. From a real colon cancer data analysis, it was elucidated that FCROS method identified additional 59 genes that were not detected by the other methods and most of them belongs to the different cancer related pathways.

Key words: Biomarker genes, DE genes, FCROS, outliers, robustness

Introduction

Microarrays gene expression data analysis is a promising area of bioinformatics. It allows simultaneous measurement of the expression levels of thousands of genes. To generate the gene expression data there are many ways, the most popular way is the so called DNA microarray technology. Microarray gene expression data can be viewed as a matrix of $m \times n$ dimension, organized by m genes versus n samples (patients) after completing several steps with the help of biological technology and statistical learning. In general m may have 10 - 100 thousands of genes and n can have 3 - 30 samples. This unique data structure has discovered as a completely new area of research for both statisticians and biologists. At the same time it provides a challenge to researchers because of high dimensionality and its complexity (large m and small n problem). The main purpose of the microarray experiments is to select the biomarker genes by comparing levels of gene expression between two different groups/conditions (Kaissi et al. 2013). In general, one group is known as reference and other is experiment. There are several methods available in the literature in this regard and many studies have been performed to select the appropriate method among these methods. For

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example Cui et al. (2003) performed a comparative study among the popular gene selection methods such as T-test, SAM-significance analysis of microarray (Tusher et al. 2001), LIMMA (Efron et al. 2001), F-test (Kerr et al. 2000) and Kruskal Wallis (KW) (Kruskal et al. 1952). Dembele and Kastner (2014) developed a new approach called fold change rank ordering statistic (FCROS) based on the FC ranks between two experimental groups. However, most of the approaches discussed above are not robust against outliers. Also, most of the comparative studies did not consider the problems of outliers in their datasets. Outliers are often occur in the gene expression data due to several steps involved in the data generating process from hybridization to image analysis (Shahjaman et al. 2017). Thus in presence of outliers, the results of downstream analysis using the popular gene selection methods might be changed. Mollah et al. (2015) reported that the assumption of normality of many gene selection methods do not hold for some existing microarray datasets in presence of outliers. Though, KW and FCROS are robust against outliers for large sample case but they are sensitive to outliers for small-sample case. Furthermore, it is very difficult to identify few biomarker genes from the high-dimensional outlying gene expression dataset and there is no precise guideline about the selection of methods. Therefore, in this paper, it was evaluated the performance among the popular biomarker gene selection statistical methods based on microarray gene expression data set in absence and presence of outliers to select the proper method.

Materials and Methods

In order to assess the performance of all the gene selection methods, we consider the different measures such as true positive rate (TPR), true negative rate (TNR), false positive rate (FPR), false negative rate (FNR) and area under the Receiver Operating Characteristics curves (AUC). In a two groups prediction problem such as DE (differentially expressed) or EE (equally expressed), the outcomes are divided into four categories: (i) truly DE that are reported as DE (True Positives: TP), (ii) truly EE that are reported as DE (False Positive: FP), (iii) truly DE that are reported as EE (False Negative: FN) and (iv) truly EE that are reported as EE (True Negatives: TN). Then the formulas of all the measures are as follows:

$$TPR = TP / (TP + FN), TNR = TN / (TN + FP), FPR = FP / (TN + FP), FNR = FN / (FN + TP).$$

Three R packages were used as (i) *limma*, which was proposed by Smyth et al. (2003), (ii) *samr*, which was developed by Tibshirani et al. (2013) and (iii) *ROCR* to obtain an area under a ROC curve (AUC) by Sing et al. (2005). A method will be called good performer that produces the higher values of TPR, TNR and AUC and lower values of FPR and FNR. We declared a gene as DE gene with adjusted p -value < 0.05 . P -values are adjusted by the Benjamini and Hochberg (1995) multiple testing correction method.

Simulated dataset

We applied five biomarker gene selection methods in the simulated dataset. The simulated dataset was generated using the following one-way ANOVA model developed by Kerr et al. (2000):

$$x_{jk} = \mu_j + \epsilon_{jk}; (j = 1, 2; k = 1, 2, \dots, n_j) \quad (1)$$

where, x_{jk} is the k th observed expression of a gene in the j th condition, μ_j is the mean of all expressions of a gene in the j th condition and ϵ_{jk} is the random error term that follows $N(0, \sigma^2)$. The outlying dataset was generated by multiplying a constant (say, 5) with the mean of equation (1).

Colon cancer real dataset

To investigate the performance of the five methods as early mentioned, in the real microarray gene expression data was used colon cancer data set was used which consists of 22 normal and 40 tumor samples. Alon et al. (1999) was used this dataset in their study. This dataset contains 2000 genes.

Results and Discussion

To investigate the performance of the five biomarker gene selection methods 100 data sets were generated from one-way ANOVA model using equation (1) for both small ($n_1 = n_2 = 3$) and large-sample ($n_1 = n_2 = 15$) cases with two groups.

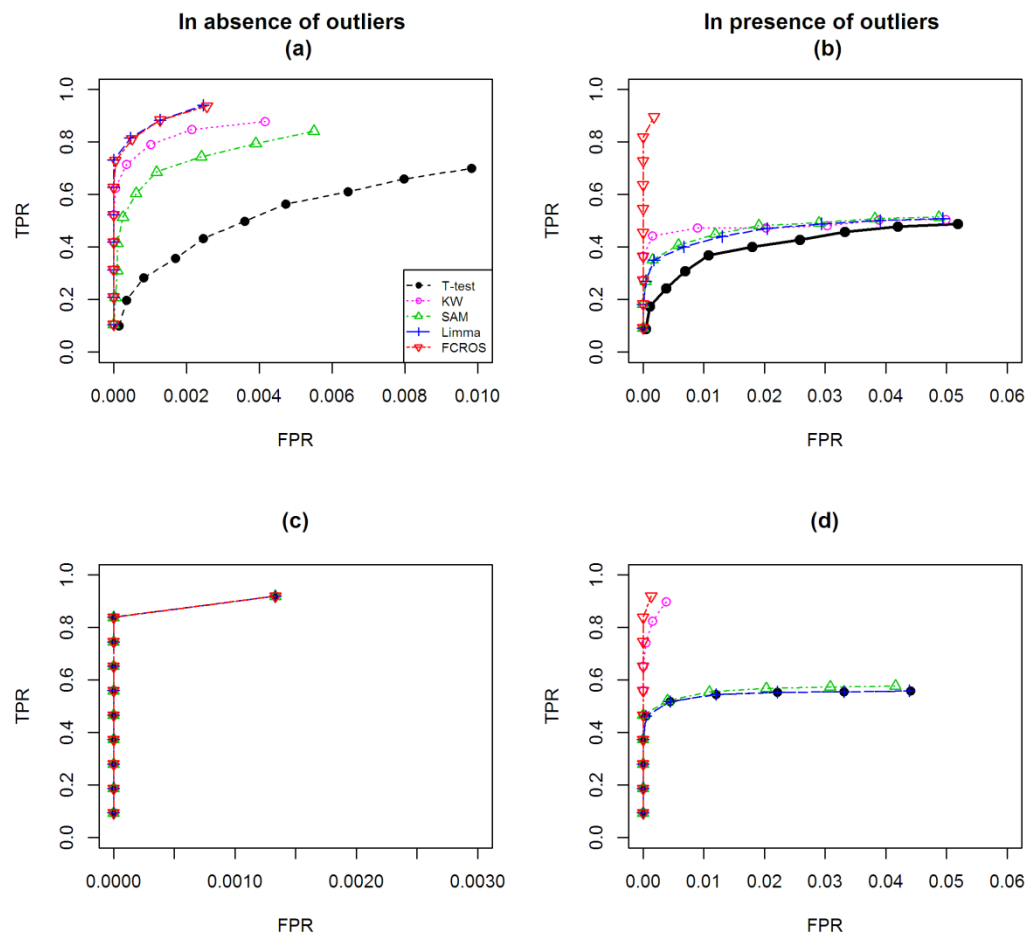


Fig. 1. ROC curve produced by different methods based on simulated gene expression data set. For small-sample case ($n_1 = n_2 = 3$): (a) in absence of outliers, (b) in presence of outliers. For large-sample case ($n_1 = n_2 = 15$): (c) in absence of outliers and (d) in presence of outliers.

In this case, 10000 genes were generated with $(n_1 + n_2)$ samples for each of the dataset. The number of DE gene is set to 300 and the rest of the 9700 genes are considered as the EE genes. The arbitrary values $(\mu_1, \mu_2) \in c(3, 5)$ and $\sigma^2 = 0.1$ were set. Each dataset for each case were represented the gene expression profiles of 10,000 genes and $(n_1 + n_2)$ samples. Fig.1a and c represents the ROC curve produced by different methods in absence of outliers for both small-sample size ($n_1 = n_2 = 3$) and large-sample size ($n_1 = n_2 = 15$), respectively. It is clear from these figures that in absence of outliers all the methods produce similar results, except T-test for small-sample case. But in presence of 5% outliers, for small-sample case ($n_1 = n_2 = 3$), the performance of all the methods has significantly deteriorated.

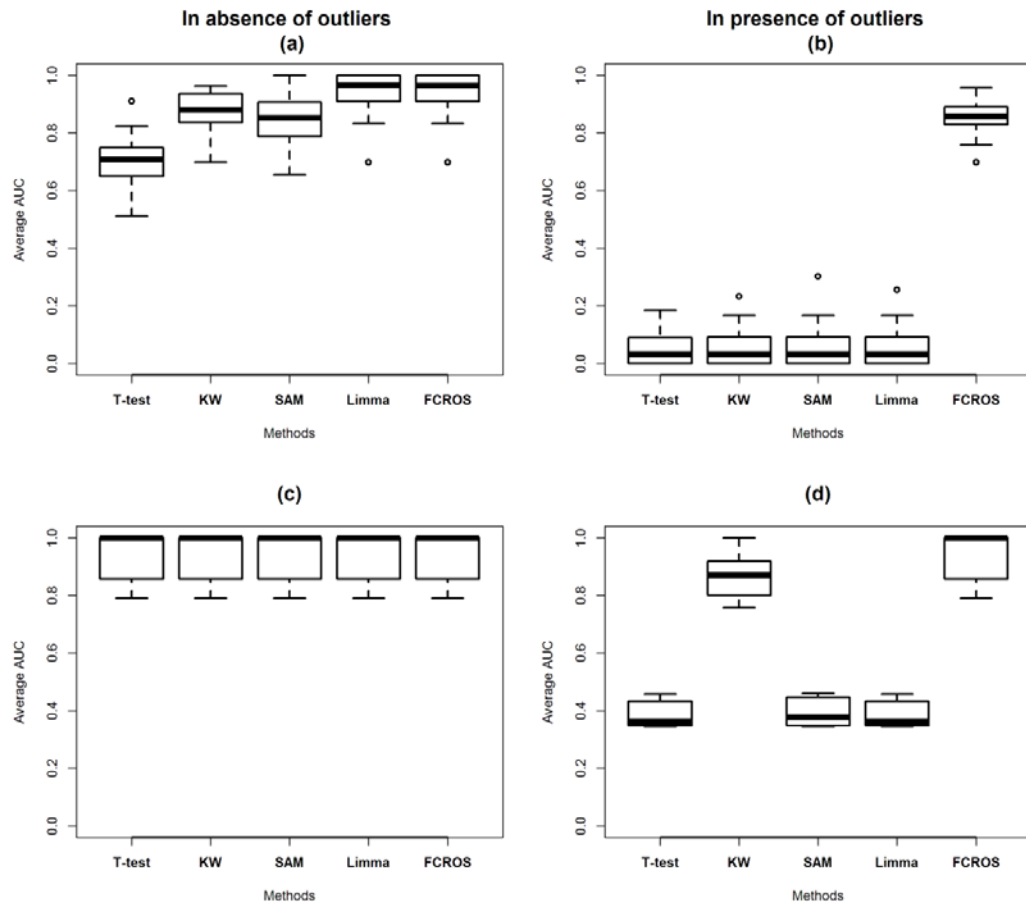


Fig. 2. Box plot of AUC values produced by different methods based on simulated gene expression data. For small-sample case ($n_1 = n_2 = 3$): (a) in absence of outliers, (b) in presence of outliers. For large-sample case ($n_1 = n_2 = 15$): (c) in absence of outliers and (d) in presence of outliers.

In this case only FCROS method performed well (Fig.1b). Whereas, for large-sample case ($n_1 = n_2 = 15$) (Fig. 1d) with 5% outliers, two methods FCROS and KW performed well. FCROS performed slightly better than KW in this case. Fig. 2a and c shows the box-plots of AUC values for each of the methods based on 100 simulated datasets generated using equation (1) in absence of outliers for small-and-large sample cases,

respectively. It is evident from Fig. 2a that, in absence of outliers for small sample case LIMMA, FCROS, SAM and KW performed better than T-test. Whereas, in case of Fig. 2c, for large sample case every methods performed similar. Fig. 2b and d represents the box-plots of AUC values based on 100 simulated datasets in presence of 5% outliers for both small-and large-sample cases respectively. It is apparent from Fig. 2b that, for small-sample case, the performance of all the methods has declined significantly except FCROS. However, for large-sample case in presence of outliers (Fig. 2d) two methods FCROS and KW performed well than the other three methods (T-test, SAM and LIMMA). Table 1 summarizes the average values of the different performance measures TPR, FPR, TNR, FNR and AUC based on 100 simulated datasets for both small-and-large sample cases in absence and in presence of outliers, respectively.

Table 1. Performance evaluation based on simulated dataset with 2 groups.

For small sample size ($n_1 = n_2 = 3$)										
In absence of outliers						In presence of outliers				
Methods	TPR	FPR	TNR	FNR	AUC	TPR	FPR	TNR	FNR	AUC
T-test	0.748	0.008	0.992	0.252	0.746	0.343	0.02	0.98	0.657	0.342
SAM	0.892	0.004	0.996	0.108	0.891	0.374	0.019	0.981	0.626	0.373
LIMMA	0.947	0.002	0.998	0.053	0.947	0.364	0.019	0.981	0.636	0.364
KW	0.860	0.005	0.995	0.140	0.860	0.351	0.020	0.98	0.649	0.351
FCROS	0.943	0.002	0.998	0.057	0.943	0.915	0.003	0.997	0.085	0.915
For large sample size ($n_1 = n_2 = 15$)										
In absence of outliers						In presence of outliers				
Methods	TPR	FPR	TNR	FNR	AUC	TPR	FPR	TNR	FNR	AUC
T-test	0.939	0.003	0.997	0.061	0.939	0.379	0.019	0.981	0.621	0.379
SAM	0.939	0.003	0.997	0.061	0.939	0.395	0.019	0.981	0.605	0.395
LIMMA	0.939	0.003	0.997	0.061	0.939	0.382	0.019	0.981	0.618	0.382
KW	0.939	0.003	0.997	0.061	0.939	0.855	0.006	0.994	0.145	0.854
FCROS	0.939	0.003	0.997	0.061	0.939	0.939	0.003	0.997	0.061	0.939

It is noticeable from this table that, for small-sample case in absence of outliers every method performed well except T-test while in presence of outliers, only FCROS performed well than the other four methods (T-test, KW, SAM and LIMMA) methods. For example, FCROS produces AUC >0.90 than T-test, KW, SAM and LIMMA (AUC <0.40). These results also reflected in the Fig. 1b. On the other hand, for large-sample case in absence of outliers all the methods performed similarly. However, in presence of outliers in this case FCROS and KW performed better comparing with the others three methods (T-test, SAM and LIMMA).

To demonstrate the performance of all the methods in the real colon cancer dataset, firstly five methods were directly applied to identify the DE genes in this dataset. DE genes were detected using adjusted p -value < 0.05 . p -values were adjusted using Benjamini-Hochberg (1995) method. Fig. 3a represents the Venn diagram of DE genes detected by these five methods. From this Venn diagram, it was revealed that LIMMA and SAM performed better than the other three methods (T-test, KW and FCROS) by sharing more genes. The number of genes that were detected as DE by T-test, KW, SAM, LIMMA and FCROS, respectively is 367, 381, 352, 372 and 383. We also noticed that there are 274 genes common between these five methods. Fig. 3b shows the heat-map plot using hierarchical clustering analysis based on the common DE genes. There are 3, 59 and 14 genes that were identified by the T-test, FCROS and KW independently. Among these genes we explored the biological functions of 59 genes detected by FCROS using a web-based gene set analysis toolkit (Zhang et al. 2005). Fig. 4 represents the bar chart of the biological process, cellular component and molecular function categories. In this figure, biological Process, cellular component and molecular function category are represented by a red, blue and green bar, respectively. Using the KEGG database, it was found that these genes were involved in different pathways (Table 2 for top 10 pathways). In this table the hyper geometric test is used to calculate the p -values.

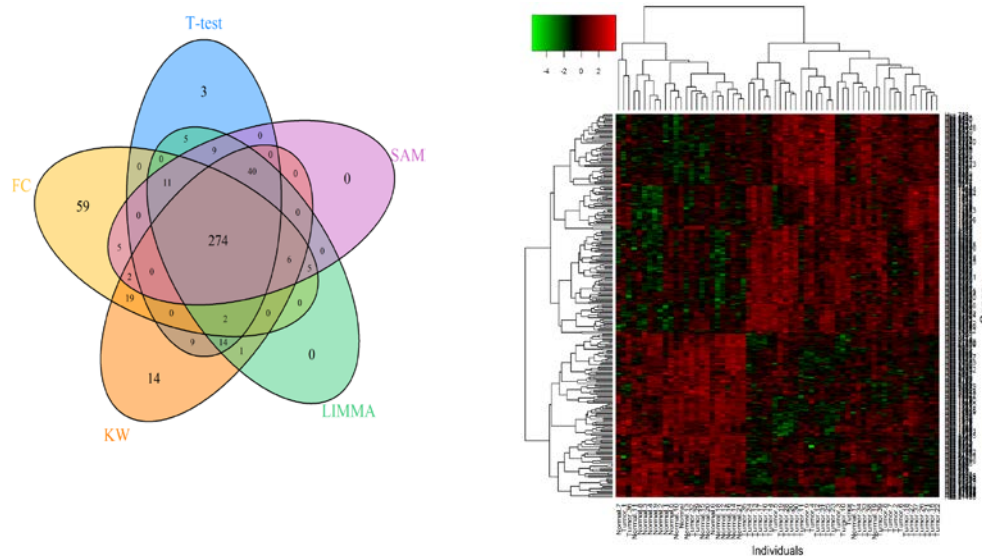
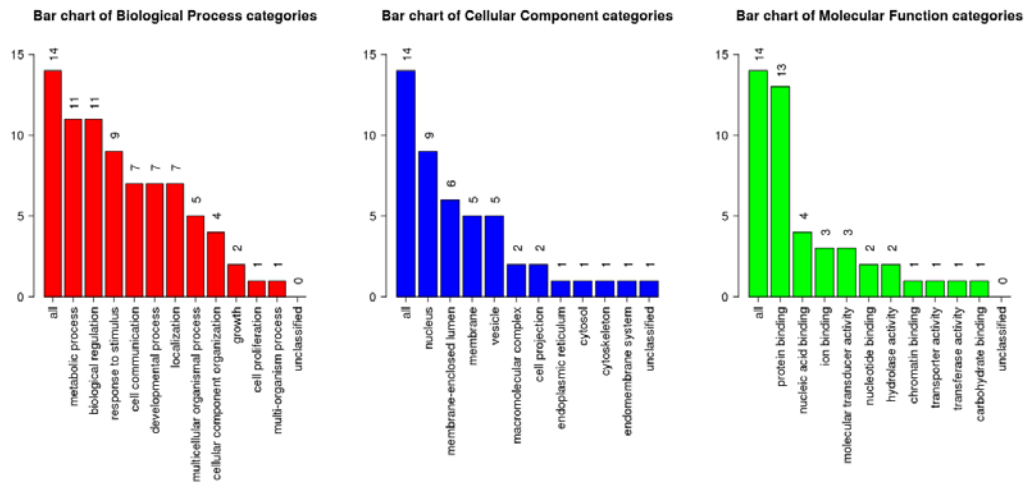


Fig. 3. Comparison of five methods for the selection of biomarker genes in colon cancer data set. (A) Venn diagram of DE genes detected by T-test, KW, SAM, LIMMA and FCROS, (B) Heat-map of 274 common DE genes detected by these five methods.

Table 2. Top 10 KEGG pathways for 59 DE genes detected by FCROS method for colon cancer data set.

KEGG ID	KEGG pathway names for <i>Homo sapiens</i> (human)	No. of genes	Adjusted - <i>p</i> -values
hsa00910	Nitrogen metabolism	10	7.02e-05
hsa00030	Pentose phosphate pathway	7	2.90e-03
hsa01230	Biosynthesis of amino acids	5	3.35e-05
hsa03018	RNA degradation	4	0.0002
hsa04931	Insulin resistance	3	0.0002
hsa01200	Carbon metabolism	3	0.0002
hsa05145	Toxoplasmosis	2	0.0001
hsa03040	Spliceosome	1	0.0001
hsa04630	Jak-STAT signaling pathway	1	0.0001
hsa05152	Tuberculosis	1	0.0001

**Fig. 4.** Functional annotation of 59 DE genes identified by the FCROS method.

Conclusion

There are several methods existing in the literature to select biomarker gene and many comparative studies have been performed to select the appropriate method. But the choice of proper gene selection method is not easy when the gene expression dataset is contaminated by outliers and there is no specific guideline so far. In this paper, we evaluated the performance among the five popular gene selection methods using both simulated and real gene expression datasets in absence and presence of outliers. From the simulated data

analysis results, it was found that, in absence of outliers for both small-and large-sample cases, all the five methods performed well (except T-test for small-sample case). But in presence of outliers, for small-sample case FCROS method out performs other four methods. On the other hand, for large-sample case, in presence of outliers, KW and FCROS perform well. From a real colon cancer data analysis, it is elucidated that, FCROS method identified additional 59 genes that were not detected by the other methods. Using the KEGG pathway analysis, it is explored that this gene belongs to the different important pathways.

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CROSS-SECTIONAL ANATOMY OF LEAF BLADE AND LEAF SHEATH OF COGON GRASS (*IMPERATA CYLINDRICA* L.)

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Abstract

Histology of leaf blade and sheath of cogon grass (*Imperata cylindrica* L.) Beauv., indicated typical C₄ Kranz anatomy. Cells of adaxial epidermis were smaller and bulliform cells were present on the adaxial epidermis. The shape of bulliform cells was bulbous; 3-7 cells were present in a group and 3-5 folds larger than epidermal cells. Three types of vascular bundles in respect of size and structure were extra large, large and small and they were part of leaf blade histology. These three sizes of vascular bundles were arranged in successive manner from midrib to leaf margin. Leaf sheath bundles were of two types: large and small. Extra large bundles were flanked by five small and four large bundles but small bundles were alternate found to be with large typed bundles. Extra large bundles were of typical monocotyledonous type but the large type had reduced xylem elements and the small typed was found to be transformed into treacherous elements. Small bundles occupied half the thickness of the flat portion of leaf blade topped by large bulliform cells of the adaxial epidermis. Extra large and large bundle had been extended to upper and lower epidermis. Kranz mesophyll completely encircled the bundle sheath and radiated out into ground tissue. Midrib was projected in abaxial direction and had a central vascular bundle with large and small bundles on either side of it along the abaxial regions. The midrib vascular bundle was devoid of chlorenchymatous bundle sheath and was of non-Kranz type. Continuous sub-epidermal sclerenchyma girders were noted as adaxial hypodermis. Anatomical traits exhibited an important adaptive defense against draught and saline stress of the plant. Quantitative measurement of various anatomical traits indicated strong variations among them.

Key words: Cogon grass, epidermis, kranz anatomy, leaf blade, vascular bundles

Introduction

Imperata cylindrica L. commonly known as Cogon grass (Bengali - 'Sone'), an aggressive, rhizomatous and weedy grass, is a troublesome weed throughout the tropical and subtropical region of the world. In Bangladesh it is conserved and even cultivated in arid lands for its use as domestic dust sweepers and roofing of thatched cottage. It is a good sand binder and possesses great adaptability to various habitats like saline coastal belt to drought hit areas of Bangladesh. It has several ecotypes (Chang and Chou 1997) of which sun and shade ecotypes are most prevalence in Bangladesh. It has been listed as one of the seven worst weeds of the world (Chang and Chou 1997). Cogon grasses grow in high temperature region and belong to the family Gramineae. It is assumed to have Kranz type leaf anatomy similar to those of C₄ plants.

A large number of graminaceous species possess Kranz anatomy, where leaf vascular bundles have two concentric and adjacent sheaths, an inner mesostome sheath of small, very thick walled, chlorenchyma lacking

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cells; and an outer parenchyma sheath of thick or thin-walled cells, with few to numerous chloroplasts and of cell sizes from average to quite large. Parenchymatous bundle sheath in most cases is associated with mesophyll cells but there are numerous exceptions of the general description (Brown 1975).

Many C₄ monocots contain suberin in the wall of the kranz cells, a feature that is absent from the eudicots (Hattersley and Browing 1981, Edwards and Vozsensenskaya 2011, Mertz and Brutnell 2014). Suberin slows diffusive efflux, thus event in the formation of the modern biosphere (Edwards et al. 2010, Christin et al. 2013, Slewinski 2013).

Biologists are now in a much better position to resolve the kranz enigma as new developmental models and genomic tools to facilitate the linkage of traits with the underlying genetic control (Covshoff et al. 2012 and 2014, Williams et al. 2013, Fouracre et al. 2014). The present study was under taken to identify Kranz system of anatomy of leaf-sheath, leaf-blade and leaf-midrib of *Imperata cylindrica* L.

Materials and Methods

Imperata cylindrica L. naturally grown in the Botanical Garden, University of Rajshahi was collected from five different regions of the gardens. Ten healthy full grown plants from each region were selected at random and full grown leaves with leaf sheath were sampled from each plant. Plant samples were collected from the middle of the leaf blade and leaf sheaths for anatomical study. Free hand transverse sections were cut for leaf sheath and leaf blade; thin sections were selected and stained with double stain technique (Johanson 1940). Cross sectional anatomy were examined through a research microscope fitted with digital camera and connected with Macintosh computer.

Gross anatomy were studied by taking microphotographs and quantitative measurements of various tissue, cells etc. were made through image analysis using *Motic j1.0* software. Collected data on the attributes analyzed through range, mean with standard deviation and tested between plants within sample area.

Results and Discussion

Transverse sections through leaf blade are shown in Figs 1 and 2. The blade exhibited Kranz anatomy characteristic of C₄ grasses (Esau 1977, Dangler et al. 1994) with mesophyll radially arranged around chlorenchymatous bundle sheath. The layers of Kranz mesophyll radially encircling successive bundles were separated from one another by a layer of non-Kranz cells, or colourless cell, containing few chloroplasts (Fig. 2).

In some observations the colourless cells resembles the bulliform cells of the adaxial epidermis and often are in contact with them. In most of the observations, the maximum lateral cells counted were two i.e. there are two Kranz mesophyll cells between chlorenchymatous bundle sheaths (Figs 3 and 4). The mesophyll cells were much branched and loosely arranged, having numerous intercellular air spaces among them.

Anatomy of the mid rib and leaf blade

As seen in any given transverse section, three types of longitudinal vascular bundles have been recognized: extra large, large and small. Three types of vascular bundles in leaf blade of grasses have been reported by Artschwager (1925) and after a long time by Ellis (1976). Commonly small bundles are alternated with large bundles. Extra large bundles (Fig. 2) are flanked by five small and four large bundles. In sugarcane, large bundles were found to be flanked by small ones or an intermediate one (Colbert and Evert 1982). Almost all of the longitudinal bundles of the blade were associated with longitudinal plates or strands of hypodermal sclerenchyma (Figs 1-3). In general the sclerenchyma was found to be strongly developed in the large vascular bundles. Moreover, the leaf margins had sclerenchyma at all levels of the blade.

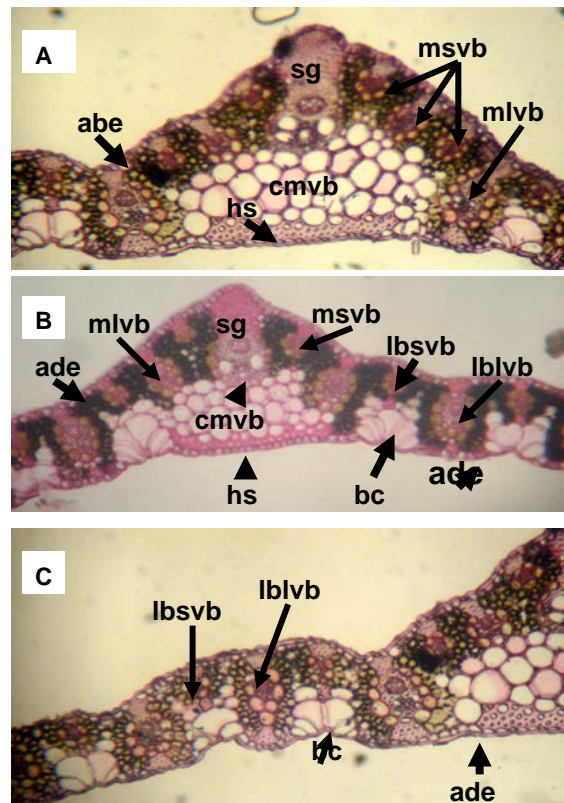


Fig. 1. Transverse section of leaf blade with midrib of cogon grass (A, B and C): abe = abaxial epidermis; msvb = midrib small vascular bundles; cmvb = central vascular bundle; hs = hypodermal sclerenchyma; mlvb = midrib large vascular bundle; bc = bulliform cell; lbsvb = leaf blade small vascular bundle; lblvb = leaf blade large vascular bundle; ade = adaxial epidermis sg= sclerenchyma girder.

The midrib shape was more or less conical by projected in abaxial direction. It consisted of a central large vascular bundle along with few small and medium bundles on either side touching abaxial epidermis (Fig. 1). In the midribs a continuous sub epidermal layer of sclerenchyma commonly formed beneath the adaxial epidermis. The central region of the midrib has colourless thin walled cells usually larger in size (Fig. 1) similar to those reported in sugarcane (Colbert and Evert 1982). The central vascular bundle of the midrib as measured was $2144.16 \mu\text{m}^2$ (ranged $1642\text{-}2471 \mu\text{m}^2$). Hameed and Ashraf (2009) reported larger size vascular bundles in Cogon grass.

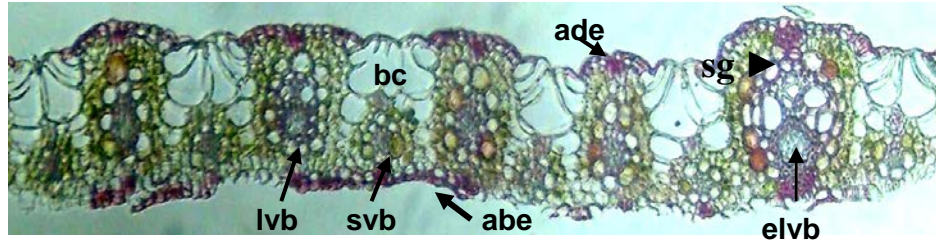


Fig. 2. Transverse section of leaf blade excluding midrib. ade = adaxial vascular bundle; bc = bulliform cell; sg = sclerenchyma girder; lvb = large vascular bundle; elvb = extra large vascular bundle; abe = abaxial epidermis; svb = small vascular bundle.

The mean inter-veinal distance as calculated from middle of one bundle to middle of another bundle was $38.64 \pm 1.64 \mu\text{m}$ in the blade and that in midrib was $27.66 \pm 3.14 \mu\text{m}$. Interveinal distance is shorter in C_4 grasses (Dangler et al.1994). In sugarcane it is variable depending on the genotypes (Elahi and Ashraf 2001). Midrib thickness as measured was $429.66 \pm 41.32 \mu\text{m}$ (range 287- 494 μm) and that in leaf blade was $196.47 \pm 14.33 \mu\text{m}$ (ranged 124 - 266 μm). Adaxial epidermis thickness of leaf sheath and midrib were $6.46 \pm 0.84 \mu\text{m}$ and $7.86 \pm 0.72 \mu\text{m}$ respectively. The abaxial epidermis thickness was measured $11.67 \pm 1.04 \mu\text{m}$ and $14.07 \pm 1.49 \mu\text{m}$ respectively in midrib and leaf blade. Hameed and Ashraf (2009) reported a very high range of those measurements depending on ecotypes and salt range in the habitat.

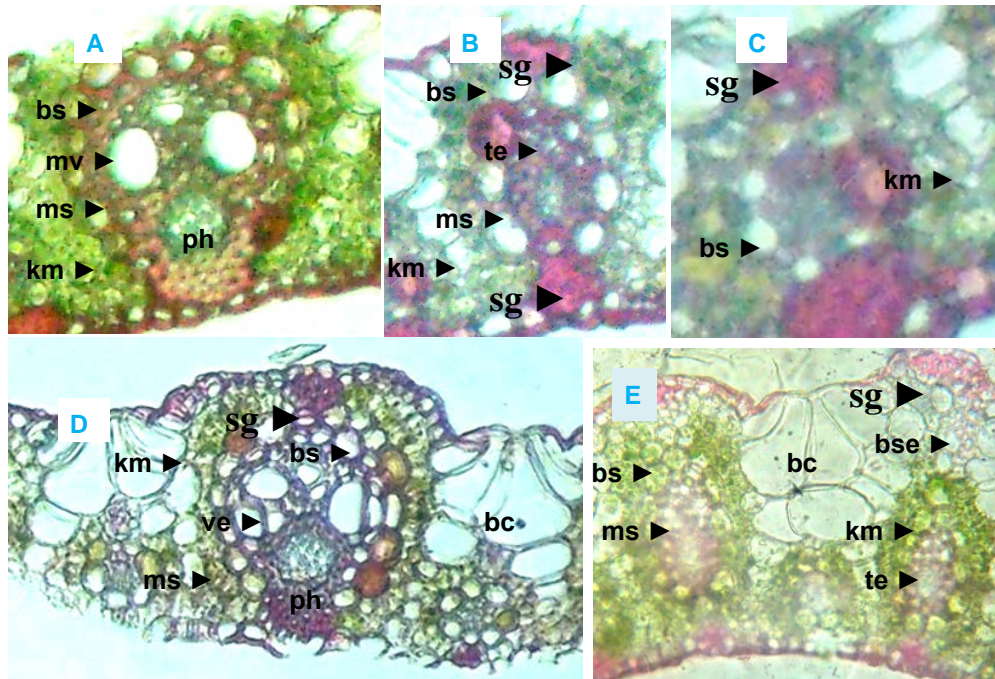


Fig. 3. Enlarged view of various vascular bundle of leaf blade. A. large bundle; B. Large VB from midrib; C. Small VB; D. Extra large VB; E. Two large bundle with a small VB. One of the large bundle developed bundle sheath extension. bs = bundle sheath; mx metaxylem vessel; ms = mesophyll sheath; ph = phloem; bc = bulliform cell; sg = sclerenchyma girder; km = Kranz mesophyll; te = tracheary element.

Table 1. Anatomical characters of midrib of Cogon grass leaf (Mean \pm standard deviation and range).

Vascular bundle at midrib (μm^2)	Interveinal distance in midrib (μm)	Midrib Thickness (μm)	Adaxial epidermis thickness of midrib (μm)	Abaxial epidermis thickness (μm)	Buliform complex area in midrib (μm^2)	Midrib vascular bundle area small (μm^2)	Midrib vascular bundle area large (μm^2)
2144.16	27.66 \pm 2.14	429.66 \pm 41.32	7.86 \pm 0.72	11.67 \pm 1.04	2896.09 \pm 141.22	1887.33 \pm 239.75	2971.17 \pm 266.44
1642-2471	25.30-30.45	287.0-494.0	7.75-8.20	10.85-12.44	2144.0-3864.0	1667.8-2045.2	2590.4-3127.4

Table 2. Anatomical characters of midrib of Cogon grass leaf (Mean \pm standard deviation and range).

Phloem area of extra large vascular bundle (μm^2)	Phloem area of large vascular bundle (μm^2)	Phloem area of small vascular bundles (μm^2)	Xylem area of large vascular bundles (μm^2)	Xylem area of small vascular bundles (μm^2)	Percentage of phloem and xylem area of large vascular bundle	Percentage of phloem and xylem area of small vascular bundle
924.64 \pm 76.124	624.87 \pm 78.32	447.44 \pm 47.24	1027.45 \pm 239.16	469.77 \pm 67.12	19.86% and 32.65%	21.89 and 22.64%
885.6-1022.2	547.4-698.0	397.5-521.7	877.0-1289.3	402.9-521.0	-	-

Table 3. Anatomical characters of leaf blade of Cogon Grass leaf (Mean \pm standard deviation and range).

Interveinal distance in leaf blade (μm)	Leaf blade thickness (μm)	Abaxial epidermis thickness (μm)	Leaf blade vascular bundle area extra large (μm^2)	Leaf blade vascular bundle area large (μm^2)	Leaf blade vascular bundle area small (μm^2)
38.64 \pm 1.64	196.47 \pm 14.33	14.07 \pm 1.49	5412.66 \pm 279.14	3146.77 \pm 271.44	2044.66 \pm 234.49
36.8-40.2	124.0-266.0	12.5-16.7	5232.5-5654.8	2970.4-323.0	1477-2697

Extra large and a few large bundles were characterized by the presence of a large metaxylem vessel on either side of the protoxylem or protoxylem lacuna (Figs 3A and D). Protoxylem was also present but in mature bundles it was usually obliterated. Protophloem not detected but active metaphloem in sieve tube and companion cells was the only conducting phloem as reported in sugarcane (Colbert and Evert 1982). Except the central midrib bundle, the large blade bundles were more or less completely surrounded by a chlorenchymatous bundle sheath, which was often interrupted on the adaxial and/or abaxial surfaces by girders of hypodermal sclerenchyma (Figs 1 and 2). An inner mestome sheath with thickened walls was always present around the phloem, and may be present around all or partly of the xylem (Fig. 3). In the midrib, the central vascular bundle lack a distinctive chlorenchymatous bundle sheath, and were associated on the abaxial surfaces with well developed sclerenchyma girders, which merged with sclerified cells subtending the phloem (Fig. 1).

The bundle sheath cells of the midrib central bundle had transformed into thick walled cells and commonly only those cells bordering the phloem had chloroplast. It is those bundle sheath cells that were in contact with Kranz mesophyll cells. The large bundles, except in midrib region, essentially were found to be extended from epidermis to epidermis (Figs 1 and 2).

Large bundles of the leaf blade (except extra large one) together with their bundle sheaths essentially were extended from upper and lower epidermis. Unlike extra large bundles, large blade bundles were lack larger

metaxylem vessels and protoxylem; such bundles were consisted of sieve tube and companion cells. Large bundles were almost completely surrounded by a chlorenchymatous bundle sheath and were associated with both adaxially and abaxially by hypodermal sclerenchyma (Figs 1- 3). The chlorenchymatous bundle sheath cells were always bordered by Kranz mesophyll (Figs 1- 3). An inner mestome sheath borders the phloem, although it may not be complete. Bundle morphology of large type in Cogon grass was similar to those of intermediate bundles of sugarcane (Colbert and Evert 1982, Elahi and Ashraf 2001).

Unlike large bundles of the blade, small bundles occupied only half the thickness of the flat portions of the blade, being close to the abaxial epidermis and topped by large bulliform cells of the adaxial epidermis (Fig. 1 and 2). The small bundles were consisted of entirely metaxylem and metaphloem (Figs 2 and 3). Small bundles were completely encircled by a chlorenchymatous bundle sheath and merged with the girder of hypodermal sclerenchyma of abaxial epidermis. Moreover the inner sheath of thick walled cells was not visible with light microscope. All the chlorenchymatous bundle sheath cells of the small blade bundles were in direct contact with the Kranz mesophyll. Lack of mestome sheath of small bundles had been reported in grasses (Carolin et al. 1973, Crookston and Moss 1973, Brown 1975) and in sugarcane (Colbert and Evert 1982).

Bulliform complex area ranged from 2144 - 3864 μm^2 in different leaves with a mean value of $2896.09 \pm 141.22 \mu\text{m}^2$. Compared to bulliform cell area, small blade bundle area ranged from 1477 - 2697 μm^2 (mean = $2044.66 \pm 234.49 \mu\text{m}^2$) in different leaves studied. Bulliform cells play an important role in leaf rolling to avoid water loss during drought stress (Abernethy et al. 1998, Balsamo et al. 2006, Alvarez et al. 2008). The presence of greatly enlarged bulliform cells is a significant adaptation against water loss under drought condition. Extensive leaf rolling was observed during summer months. Hameed and Ashraf (2009) in Cogon grass and Arton (1986) in some species of *Axonopus* observed the similar phenomenon.

Leaf blade vascular areas were measured and it was $5412.66 \pm 279.14 \mu\text{m}^2$, $3146.77 \pm 271.44 \mu\text{m}^2$ and $2044.66 \pm 234.49 \mu\text{m}^2$ for extra large, large and small bundles respectively. Midrib bundles (except central vascular bundles) as measured were $1887.33 \pm 239.75 \mu\text{m}^2$ and $2971.17 \pm 266.44 \mu\text{m}^2$, respectively for small and large bundles. Hameed and Ashraf (2009) reported very high estimates of vascular bundle area of Cogon grass.

Phloem area of the extra large vascular bundle was $924.66 \pm 76.12 \mu\text{m}^2$. The xylem area was $2416.33 \pm 149.77 \mu\text{m}^2$. Phloem and xylem area were consisted of 17.08% and 44.64% of the vascular bundle area, respectively. Smaller phloem size compared to xylem area has been reported in Cogon grass by (Hameed and Ashraf 2009). High standard error attached with the mean of phloem and xylem area is indication of high range of variation existed in different sample area. Hameed and Ashraf (2009) noted high variation in vascular bundle traits in ecotypes of Cogon grass grown in different range of salt level. Phloem area of large and small bundles were $624.87 \pm 78.32 \mu\text{m}^2$ and $447.44 \pm 47.24 \mu\text{m}^2$, and these for xylem were $1027.45 \pm 239.16 \mu\text{m}^2$ and $469.77 \pm 67.12 \mu\text{m}^2$, respectively. Percentage of phloem and xylem area of large vascular bundle over large vascular bundle area was 19.86% and 32.65%; and those for small bundles were 21.89% and 22.64%, respectively.

Anatomy of sheath

Transverse sections of leaf sheath are shown in Figs 4 and 5. Leaf sheath thickness was greater in the midpoint and tapered towards the margin. The midpoint was round in contrast to midrib (Fig. 4). Leaf sheath also exhibited Kranz anatomy as in leaf blade. Sugarcane leaf sheath do not have Kranz anatomy (Colbert and Evert 1982). It was comprised of a large vascular bundle flanked by 2-3 small bundles of Kranz type. Central bundle have two large metaxylem vessels and protoxylem (Figs 5 and 6).

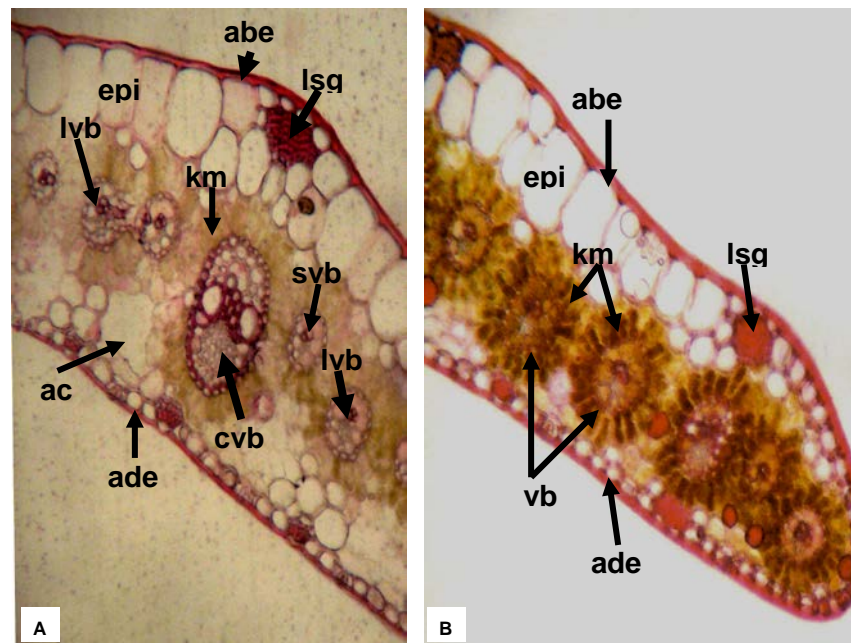


Fig. 4. Transverse section through leaf sheath. A. section through middle of mid region equivalent of midrib of blade, B. Section through margin of sheath. abe = abaxial epidermis; lsq = large sclerenchyma girder; epi = epidermis; km = kranz mesophyll; lvb = large vascular bundle; cvb = central vascular bundle; ac = air cavity; ade = adaxial epidermis; vb = vascular bundle in the margin.

Phloem was large and well developed. As seen in transverse sections, the pattern of longitudinal bundles in the sheath was more regular than that in blade. Beginning at the margin there were usually one or two small bundles (Fig. 4) followed by uniformly arranged fairly large bundles of equal size. The bundles in the margin were measured as $714.49 \pm 31.34 \mu\text{m}^2$ and next to these as measured as $945.66 \pm 41.41 \mu\text{m}^2$. The large centre bundle as measured was $1424.67 \pm 149.11 \mu\text{m}^2$. Leaf sheath bundle area as measured in Cogon grass was very high as reported and ranged from $5919\text{--}8282 \mu\text{m}^2$ (Hameed and Ashraf 2009). These differences with our study could be due to ecotype differences. Central mid bundle and most bundles in the margin are associated with fairly large amounts of sclerenchyma, particularly on their abaxial surface (Fig. 4).

Adaxially a vascular bundle may or may not be contiguous to a sclerenchyma strand. Sclerenchyma thickness as measured $16.69 \pm 0.94 \mu\text{m}^2$, which was largest in abaxial surface but it was $7.14 \pm 0.19 \mu\text{m}^2$ in adaxial side. Ecotype and habitat influenced sclerenchyma thickness in Cogon grass (Hameed and Ashraf 2009). Leaf sheath bundles together with their bundle sheath and kranz mesophyll essentially extended from upper to lower epidermis (Fig. 5). The abaxial epidermal cells were very large, radially elongated, and each measured $343.55 \pm 34.11 \mu\text{m}^2$. The adaxial epidermal cells were small in size and each measured as $8.19 \pm 0.24 \mu\text{m}^2$. Hameed and Ashraf (2009) reported larger abaxial and smaller adaxial epidermal cell area in Cogon grass. A clear large air cavity was observed in the leaf sheath midpoint which measured $624.22 \pm 47.66 \mu\text{m}^2$. Presence of aerenchyma in Cogon grass has been reported by Hameed and Ashraf (2009).

Table 4. Anatomical characters of leaf sheath of Cogon Grass (Mean \pm standard deviation and range).

Leaf sheath in the margin small bundle1 (μm^2)	Leaf sheath in the margin small bundle2 (μm^2)	Leaf sheath centre bundle (μm^2)	Adaxial epidermis thickness in leaf sheath (μm)	Adaxially vascular bundle sclerenchyma thickness (μm^2)	Abaxially vascular bundle sclerenchyma thickness (μm^2)	Abaxially epidermal cells large (μm^2)	Abaxially epidermal cells small (μm^2)	Leaf sheath mid point air cavity (μm^2)
714.49 \pm 31.34	945.66 \pm 41.41	1424.7 \pm 149.11	6.46 \pm 0.84	16.69 \pm 0.94	7.14 \pm 0.19	343.55 \pm 34.11	8.19 \pm 0.24	624.22 \pm 47.66
690.3-748.2	902.6-980.6	1332.4-1580.7	5.80-7.35	15.45-17.28	7.05-7.22	305.5-376.5	7.55-8.80	578.9-690.3

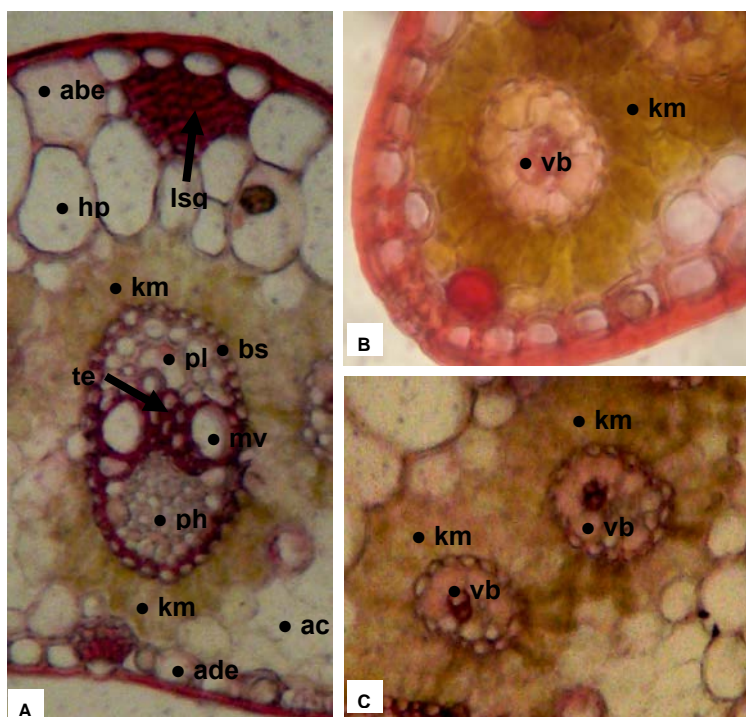


Fig. 5. Enlarged view of cross section of leaf sheath. A. Section from widest region, B. Section through margin and C. section from away margin. abe = abaxial epidermis; lsg = large sclerenchyma girder; hp = hypodermis; km = kranz mesophyll; bs = bundle sheath; te = tracheary elements; ph = phloem; ac = aerenchyma; ade = adaxial epidermis; mx = metaxylem; vb = vascular bundle.

Leaf blade and leaf sheath anatomy provided good information on C_4 type photosynthesis which operates in both blade and sheath. Some special adaptation like tolerant to harsh environment like well development leaf rolling system, hypodermal sclerenchyma; and aerenchyma complex were well expressed. This result could be targeted to modern molecular and genetic engineering technique for utilization of this adaptation to other crop plants. High standard error attached with various measurements indicated plant to plant variation within a selection cite is very high. It is an indication of presence of the different ecotypes in the sample area.

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STUDIES ON ANTIOXIDANT POTENTIAL, PHYTOCHEMICAL PROPERTIES AND TOXICITY OF FOUR POPULAR MEDICINAL PLANTS OF BANGLADESH

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Abstract

Medicinal plant extract has long been used successfully in 'unani' or 'ayurvedic' medicine. Medicinal plant extract contains bioactive molecules and activity of these molecules may help to mitigate, eradicate or cure diseases. In the advent for the search for new medicinally important bioactive molecule, the current paper deals with the anti-oxidative, cytotoxic and phytochemical analysis of *Scoparia dulcis* leaf and root, *Curculigo orchoides* root, *Pandanus fascicularis* root and *Baccaurea sapida* leaf extract. Aqueous and methanolic extracts were made for each of the extracts where they possess significant antioxidative properties. High activities were seen in *P. fascicularis* and *S. dulcis* plant extract where IC₅₀ values were 21.87 µg/ml and 173.36 µg/ml respectively. In toxicity test, only *P. fascicularis* extracts showed lethality in a dose-dependent manner where the LD₅₀ value was 25.64 µg/ml. By the phytochemical analysis, it was found that each of these plant species possesses glycosides, protein, carbohydrates, alkaloid, flavonoid etc. which are pharmacologically active biomolecules. These important properties of those plants showed an indication that these plants can further be tested for the utilization in therapeutic purpose or in cosmetic industry.

Key words: Antioxidant, cytotoxicity, extracts, phytochemicals, plant

Introduction

A medicinal plant is a plant that is used in maintaining health, to be administered for a specific condition or both whether in modern or in traditional medicine (Smith-Hall et al. 2012, Ahn 2017). Plants have been utilized as medicines for thousands of years (Samuelsson 2009). These medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations (Balick and Cox 1996). However, with the advent of chemically or artificially synthesized drugs, these medicinal plants as herbal formulations declined popularity day by day. But the consequence of severe side effects of the synthetic drugs, modern drug discovery is retuning towards the nature and medicinal plants serve as gold mine for bioactive lead drug molecule (Rates 2001). Medicinal plants have been the single most productive source of leads for the development of drugs. A statistics shows that in recent years there were over a 100 new products in clinical development, particularly as anti-cancer and anti-infective agents (Harvey 2008). According to WHO, there are 21,000 medicinal plants possessing potentiality that can be used to reduce the human disease (WHO 2000).

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In Bangladesh, more than 500 plants have so far been enlisted as medicinal plant (Ghani 1998, Nishat et al. 2002). Around 400 government nursery and some private organization produce and sale medicinal plants and plant based products in Bangladesh. As the demand of medicinal plants and plant based products are increasing day by day; their cultivation has been started in different district in our country. A great example of this exploration is "Natore Owshodhi gram". This village is located at the Laxmipur Kholabaria Union under the Natore sadar upazilla District. Farmers of this village and nearby villages cultivate several medicinally important plant species as cash crops (Sharmin 2004). Despite the medicinal value of these plants very few studies were conducted regarding their phytochemical properties, antioxidant potential and cytotoxicity. Therefore, in the present study antioxidant potential, phytochemical properties and cytotoxic activity were examined for *Scoparia dulcis* leaf and root, *Curculigo orchoides* root, *Pandanus fascicularis* root and *Baccaurea sapida* leaf. As the four medicinally important plant species are the major focus paper, it will be rationale to provide a brief description of these plants below:

Baccaurea sapida

B. Sapida (Latkan) is a delicious fruit normally grown in Bangladesh, Nepal, India, Myanmar, China, Thailand and Peninsular Malaysia (Alam 2004). Latkan fruits are yellow, velvety, 2-3 cm in diameter with leathery pericarp. Seeds are arillus, three in number, embedded in pale rose colored delicious pulp. *B. sapida* fruits are used as food and in treatment of bacterial infectious diseases such as diarrhea, dysentery, skin infection (Jain 1986, Mann et al. 2008, Deb and Bhowmick 2013)

Pandanus fascicularis

Pandanus roots are used in unani and other folk medicine. Roots, leaves, fruits etc are used as anthelmintic, tonic, in treatment of liver disorders (Jothimani et al. 2011). Root extract also have anti-inflammatory and analgesic activity. In treatment of diabetes, *Pandanus* roots are used. Roots are widely used in treatment of osteoarthritis and skin diseases like leprosy. In the Ayurvedic system of medicine, the roots of *P. fascicularis* are used in 'Prameha' and employed for their hypoglycemic action (Madhavan et al. 2008, Ayyanar and Ignacimuthu 2011, Jothimni et al. 2012).

Curculigo orchoides

This plant is endangered species and it is native to Indian sub-continent including Bangladesh. This plant contains curculigoside A, B, C and D and curculigine A and D can also be found. This plant is used for diseases of the urogenital system in both males and females (Chauhan et al. 2007). It is also prescribed in treatment of piles, jaundice, gonorrhea, asthma, and diarrhea (Chauhan et al. 2010). This plant extract is present in several herbal formulations for gynecological problems and sexual weakness of males (Duraipandiyar et al. 2006).

Scoparia dulcis

S. dulcis is a weed in many parts of Bangladesh but its use as traditional medicine has led to overexploitation of this plant (Mollik et al. 2010). Scoparinol, scoparic acid, scopadulcic acid, scopadulciol, and scopadulin have been isolated from this plant. This plant is used for diabetes, hypertension and other health conditions such as hemorrhoids, anemia, burns, and headaches (Edeoga et al. 2006).

Materials and Methods

Collection of plant material

Roots of *P. fascicularis*, *C. orchoides* and leaves of *S. dulcis* were collected from the Natore Owsodhi gram. Leaves of *B. Sapida* were obtained from Narshingdi where this plant is cultivated in commercial level.

Extraction of phytochemicals

For the extraction of phytochemicals, the leaf and root of the *selected plants* were dried at room temperature and was crushed into powder by electric blender (Pant et al. 2017). For methanolic extraction, powders were extracted in methanol apparatus at 55-85°C for 12-24 h. The extracts obtained were then dried using rotavapor drier at 55-85°C, and the solid extracts were preserved in refrigerator for further analysis. For aqueous extraction powders were dissolved in dH₂O and centrifuged at 8000 rpm for 10 min at room temperature and supernatant served as crude aqueous extract.

In vitro antioxidant assay

In order to examine the antioxidant properties of methanolic extracts of the test plants explants, DPPH free radical scavenging assay (Hatano et al. 1988) was employed. DPPH antioxidant assay is based on the ability of 1, 1 diphenyl-2-picryl-hydrazyl (DPPH) a stable free radical, to decolorize in the presence of antioxidants. The percentage (%) inhibition activity was calculated from the following equation

$$\%I = \{(A_0 - A_1)/A_0\} \times 100$$

Where,

A₀ is the absorbance of the control, and

A₁ is the absorbance of the extract/standard.

Then % inhibitions were plotted against log concentration and from the graph IC₅₀ was calculated. Extracts (30 mg) of four plant samples were taken in four 1.5 ml centrifuge tube. Methanol (1 ml) was added to each of the tubes with the help of micropipette. Then the tubes were vortexed for 2-3 min so that plant extracts could mix with methanol. Subsequently, tubes were centrifuged at 10000 rpm for 5 minutes. Methanol (1.5 ml) solution and DPPH solution (1.5 ml) was then added in glass test tubes. Each test tube contained different concentrations of extract solutions (50 µg/ml, 100 µg/ml, 200 µg/ml, and 400 µg/ml). The negative control contained only methanol and DPPH solution, in contrast the positive control contained Ascorbic acid standard with methanol and DPPH was added. All the test tubes were incubated at room temperature for 30 min in dark chamber to complete the reaction. Finally, the absorbance of the solutions was measured at 517 nm wavelength using spectrophotometer.

Phytochemical properties

Phytochemical analysis was conducted using standard protocols (Obadoni and Ochuko 2002, Senguttuvan et al. 2014, Pant et al. 2017). Plants extracts were screened for glycosides, protein, carbohydrates, alkaloids, tannin, phlobatannin, flavonoid, steroids, saponins, phenolic compound, phytosterols, fats and for anthraquinones.

Fehling's test for glycosides

A small amount of grinded plant sample was taken and dissolved in water. Fehling's solution was added in test tube containing water dissolved plant crude extracts. Brick-red precipitation formation indicated the

presence of glycosides in the plant sample. In another test grinded plant sample was dissolved in methanol and few drops diluted H_2SO_4 was added. Then test tubes were boiled after adding NaOH solution for neutralization. Brick-red precipitation indicated the presence of glycosides.

General test for glycosides

Small amount of grinded plant sample was dissolved in water and a small portion of aqueous NaOH solution was added. A yellow color formation was considered the indicative of the presence of glycosides.

Biuret test for protein

First Biuret reagent was prepared according to Gornall et al. (1949). Few drops of Biuret reagent were added in four test tubes which containing different crude extracts. Upon completion of the reaction deep purple/lilac color formation confirms the presence of protein.

Benedict's test for simple carbohydrates

Different grinded plant sample (1 gm) was dissolved in water (10 ml) and filtered using Whatman 1 filter paper. From the stock solution, 5 ml were taken to other test tubes and added 5 ml Benedict reagent (Benedict 1909). Tubes were heated on to the boiling water bath for few minutes. A color change indicated the presence of simple carbohydrate.

Hager's reagent for alkaloid test

A few amount of water solution of different plant samples were neutralized by adding diluted H_2SO_4 in few drops. The solution was then treated with a small amount of Hager's reagent. The presence of alkaloid was confirmed by the formation of yellow crystals.

Ferric chloride test for tannin

Water and methanolic extracts of four plant samples were stirred in dH_2O . Then 5% FeCl_3 solution was added in tubes. The presence of tannin was confirmed by the colored (blue, green, blue-black) precipitation.

Test for phlobatannin

5 ml of aqueous and methanolic solution of four plant extracts taken and added diluted HCl. The presence of phlobatannin was confirmed by red or reddish color precipitation formation.

HCl test for flavonoid

Aqueous and methanolic solutions of plants extracts were taken and concentrated HCl were added from the side of the test tubes. Presence of flavonoid is indicated by red color formation.

Salkowski's test for steroids

Aqueous solution (5 ml) of extracts were taken in test-tubes and chloroform (3 ml) and concentrated H_2SO_4 (2 ml) were added from the side of the test tubes. Red color production in the chloroform layer is the confirmation of presence steroids.

Frothing test for saponine

Solution (5 ml) of crude aqueous extracts of different plant samples was shaken strongly in a test tube. Produced of frothing and persistence of the froth upon warming for 2-3 minutes can be taken as a primary confirmation for saponine presence.

Test for phenolic compound

1gm of grinded plant sample was dissolved in 10 ml dH₂O. Small amount of neutral 5% ferric chloride solution was added to it in test tubes. Presence of phenolic compounds in the sample was detected based on the formation of a dark green color.

Test for phytosterols

0.5 gm grind sample was dissolved in acetic anhydride solution (2 ml). Few drops of concentrated H₂SO₄ was added very slowly by the side of the test tubes. A change in color detects the presence of phytosterols.

Ethanol emulsion test for fats

Solution (5 ml) of plant extracts was taken in different test tubes. Ethanol (2 ml) were added to each test tube and mixed by gentle shaking. Upon shaking, 2 ml dH₂O were added. Formation of emulsion was manually visualized to detect the presence of fat.

Test for anthraquinones

Powder (1 gm) of grinded plant sample was taken in different test tubes. Chloroform (5 ml) was added to each of the tubes and kept on boiling water bath for 5 min. After 5 minutes of boiling, remaining solutions were filtered and cooled at room temperature. Subsequently, 10% ammonia solution (5 ml of) was added to the filtrate. The tubes were shaken to visualize the formation of bright pink color as the indicator of the presence of Anthraquinones.

Toxicity assay

Brine Shrimp lethality assay was performed using the method of Meyer et al. (1982) with *Artemia salina* (nauplii) (24 hr post hatching). Ten organisms were used for each concentration. Different concentrations of solutions were prepared using different plant extracts and dose concentration was 5 µg/ml, 10 µg/ml, 15 µg/ml, 20 µg/ml and 25 µg/ml, respectively. Reading for lethality of the nauplii was obtained after 24 hours of given doses. Percentage (%) of inhibition was calculated by comparing four samples with the control samples which do not contain any plant extract.

Results and Discussion**Antioxidant potential**

In the present study, the antioxidant activity of the plant extracts of *B. sapida*, *P. fascicularis*, *C. orchoides* and *S. dulcis* were evaluated by DPPH free radical scavenging assay. The antioxidant activity was increased by increasing the concentration of the extract in a dose dependent manner. Different concentrations of the sample extracts viz. 50 µg/ml, 100 µg/ml, 200 µg/ml and 400 µg/ml were used in the experiment. *B. sapida* extracts showed the scavenging value of 12.90%, 30.74%, 44.59%, and 57.31% respectively in four different concentration with a IC₅₀ value of 302.16 µg/ml which means moderate free-radical scavenging activity are shown by this plant extracts. However, high antioxidant activity was observed in case of *P. fascicularis* extracts with IC₅₀ value of 121.87 µg/ml. The scavenging value of the extracts were 62.57%, 66.16%, 67.30%, 69.75 % respectively in four different concentrations mentioned above. 4.14%, 13.91%, 31.77% and 58.65% of scavenging value was obtained for *C. orchoides* extracts in 50 µg/ml, 100 µg/ml, and 200 µg/ml

and 400 $\mu\text{g/ml}$ concentrations, respectively. The calculated IC_{50} value of *C. orchoides* extract was 336.98 $\mu\text{g/ml}$. In case of *S. dulcis* extracts scavenging value of 35.18%, 63.67%, 65.97%, 69.02% respectively were obtained. *S. dulcis* root extracts showed a higher free-radical scavenging activity and the IC_{50} value was 173.36 $\mu\text{g/ml}$. Antioxidant potentiality of each of the extract was compared with the standard ascorbic acid. The results of DPPH radical scavenging assays of ascorbic acid (standard) and our four plant extracts are given in Table 1 and in the Figs 1 and 2.

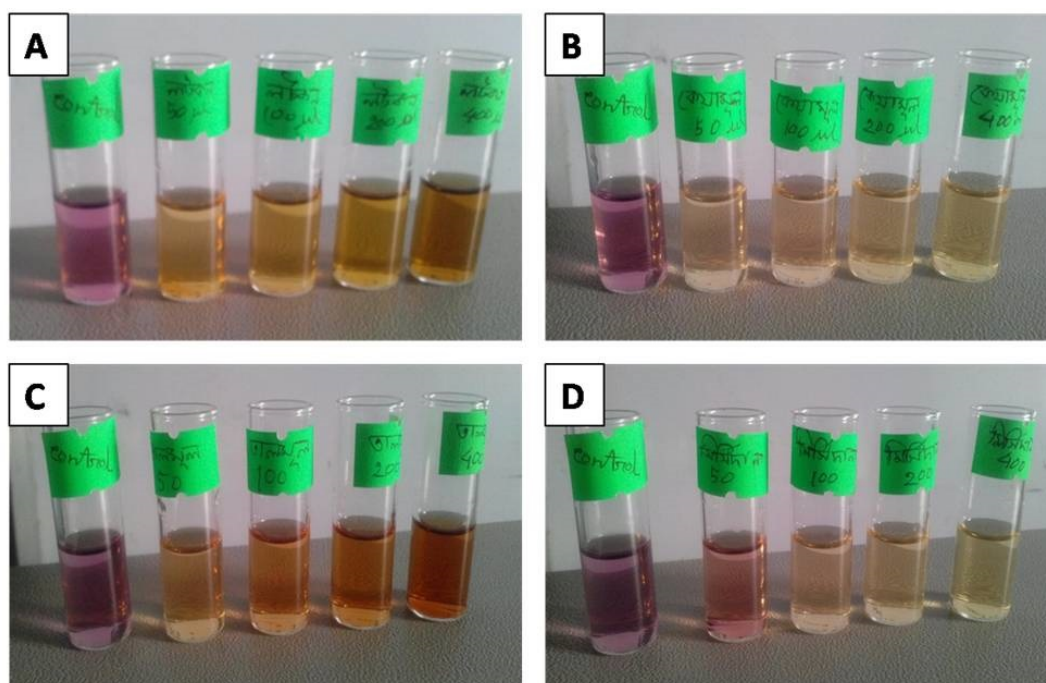


Fig.1. Decolorization of DPPH solution by (A) *B. sapida* extracts, (B) *P. fascicularis* extracts, (C) *C.orchoides* extracts and (D) *C. orchoides* extracts.

Table 1. Percentage (%) of scavenging by DPPH free radical scavenging assay of four (4) different plant sample extract along with the standard ascorbic acid.

Concentrations ($\mu\text{g/ml}$)	% of scavenging				
	Ascorbic acid	<i>B. sapida</i> (leaf)	<i>P. fascicularis</i> (root)	<i>C. orchoides</i> (root)	<i>S. dulcis</i> (leaf)
0	0	0	0	0	0
50	68.59	12.90	62.57	4.14	35.18
100	72.64	30.74	66.16	13.91	63.67
200	77.45	44.59	67.30	31.77	65.97
400	78.23	57.31	69.75	58.65	69.02

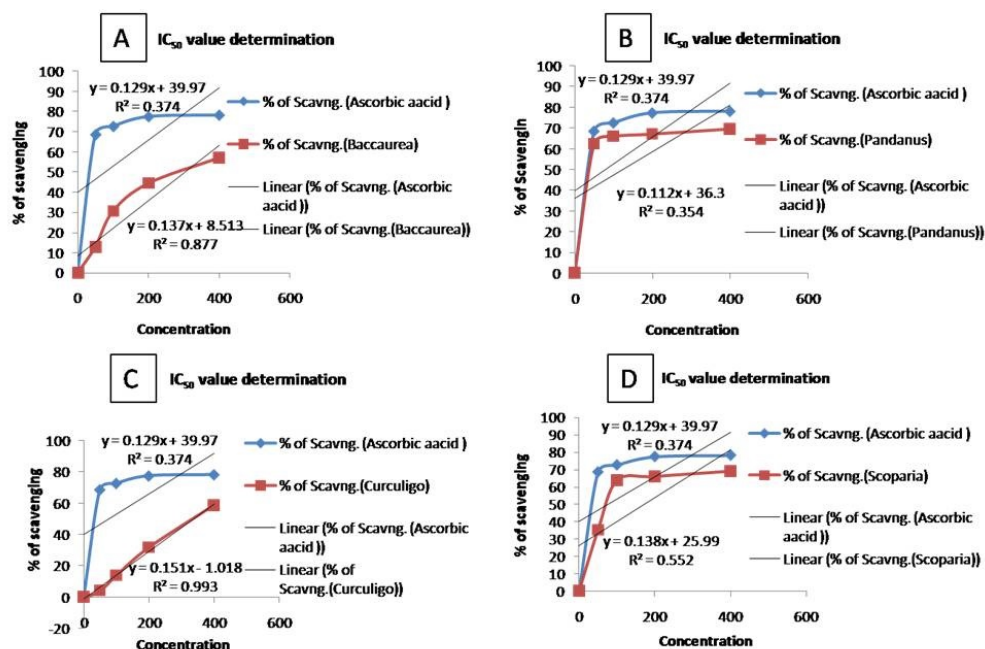


Fig. 2. DPPH free radical scavenging activity of the plant extracts at different concentrations along with ascorbic acid standard ($\mu\text{g/ml}$) (A). *B. sapida* leaf extract, (B). *P. fascicularis* root extract, (C). *C. orchoides* root extract, and (D). *S. dulciss*.

Phytochemical properties of four plant extracts

14 phytochemical properties of medicinally important four plant extracts were investigated in the present study and the results of the study are summarized in the Table 2.

Toxicity test of four plant extracts

Brine-shrimp lethality bioassay was conducted at five different concentrations (5 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, 15 $\mu\text{g/ml}$, 20 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$). Methanolic extracts of four plant extracts were used. *Baccaurea* leaves, *Scoparia* leaves, *Curculigo* roots extracts did not show any cytotoxic activity in any of those five different concentrations. Only *Pandans fascicularis* extracts showed cytotoxicity in a dose dependent manner (Table 3). From the percentage (%) lethality of brine-shrimp, probit analyses were carried out by probit analysis software (Fig. 3). From the dose value, lethal concentration 50 or LC_{50} value were calculated. LC_{50} value of *P. fascicularis* root of methanolic extract was found 25.64 $\mu\text{g/ml}$ with 95% confidence limits. The results suggest that the *P. fascicularis* root extract is highly toxic to cells at least against brine-shrimp larvae.

Aqueous and methanolic extracts of the four plant extracts were used for the qualitative analysis of the four plant extracts. *Baccaurea* leaves showed positive results for the test of presence for glycosides, carbohydrate, steroids, phenolic compound and phytosterols but negative the presence of protein, alkaloid, tannin, phlobatannin, flavonoid, saponine, fats and anthraquinones for both the aqueous and methanolic extracts.

Table 2. Qualitative analysis of the phytochemical screening of aqueous and methanolic extracts of four medicinally important plant samples from Bangladesh. Here, "+" refers to presence and "-" refers to absence.

Compound name	<i>B. sapida</i> (leaf)		<i>P. fascicularis</i> (root)		<i>C. orchoides</i> (root)		<i>S. dulcis</i> (leaf)	
	Aqueous extract	Methanolic extract	Aqueous extract	Methanolic extract	Aqueous extract	Methanolic extract	Aqueous extract	Methanolic extract
Fehling's test for glycosides	+	+	–	–	+	–	+	–
General test for glycosides	+	+	–	–	+	–	–	–
Biuret test for protein	–	–	+	–	–	–	+	–
Benedict's test for simple carbohydrate	+	+	+	+	+	+	+	+
Hager reagent for alkaloid test	–	–	–	+	–	+	–	+
Ferric chloride test for tannin	–	–	+	–	+	+	–	–
Test for phlobatannin	–	–	–	–	–	+	–	–
HCl test for flavonoid	–	–	+	–	–	–	–	–
Salkowski's test for steroids	+	+	+	–	–	–	–	–
Frothing test for saponine	–	–	–	–	–	–	+	–
Test for phenolic compound	+	+	+	+	+	+	–	–
Test for phytosterols	+	+	–	–	–	–	+	–
Ethanol emulsion test for fats	–	–	–	–	–	–	+	–
Test for Anthraquinones	–	–	–	–	–	–	–	–

In case of *Pandanus fascicularis* root extracts, for both aqueous and methanolic extracts negative result was found for the test of presence for glycosides, phlobatannin whereas the test of presence for carbohydrate was positive. In case of test of presence for protein, tannin and flavonoid, the aqueous extracts showed positive but methanolic extracts showed opposite. Negative results were found for the aqueous extracts of the test of presence for saponine, phytosterols, fats and anthraquinones.

Curculigo roots showed positive results for both the extracts for the test of presence of carbohydrate, tannin and phenolic compounds but negative for the protein, flavonoid, saponine and fat. The aqueous extracts of the *Curculigo* roots contains glycosides but not the alkaloid, phlobatannin, steroids, phytosterols and anthraquinones. Only the methanolic extracts of the *Curculigo* roots contain the alkaloid and phlobatannin.

The aqueous extracts of *Scoparia* leaves showed positive results for the test of presence for glycosides, protein, carbohydrate, saponine, phytosterols and fats but negative results for the general test for glycosides, alkaloid, steroids and anthraquinones. On the other hand, the methanolic extracts of the roots indicated negative results for the presence of glycosides, protein, tannin, phlobatannin, flavonoid, saponine, phenol and fats but positive for carbohydrates and alkaloids.

Table 3. Brine-shrimp cytotoxicity of the methanolic extract of *P. fascicularis* root.

Dose μg/ml	Log dose	Number	Kill	%kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
5	0.6989628	10	1	10	10	3.72	3.343838	3.89	2.08	3.328856
10	0.9999897	10	0	0	0	0	4.042734	3.34	4.390001	4.037427
15	1.176079	10	4	40	40	4.75	4.451563	4.78	5.580001	4.451915
20	1.301017	10	4	40	40	4.75	4.741631	4.74	6.16	4.745999
25	1.397926	10	5	50	50	5	4.966625	4.99	6.34	4.974107

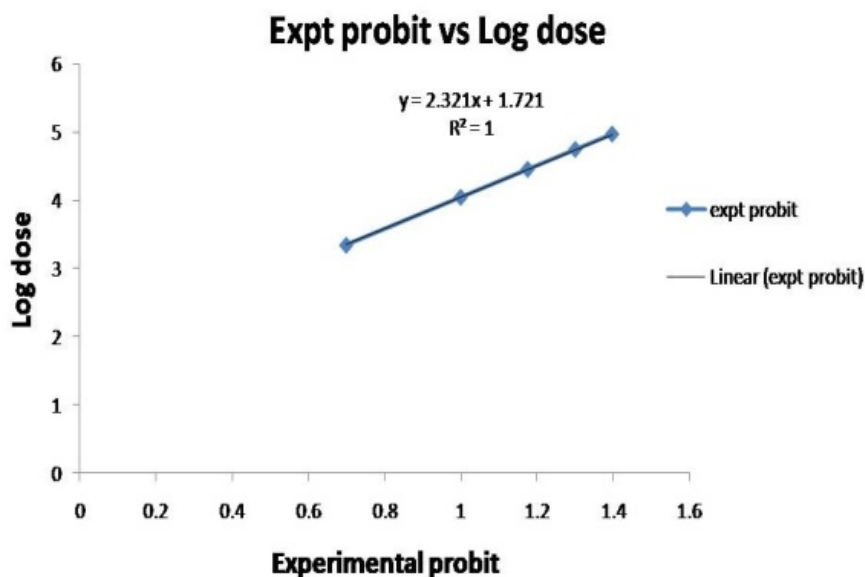


Fig. 3. Plot of log doses versus probit for calculation of LC_{50} of methanolic extract of *P. fascicularis* root. Finney's statistical method of probit analysis was used for the analysis of the data.

Conclusion

The results of the study demonstrate that each of the four medicinally important plant extract has antioxidant activity where *P. fascicularis* and *S. dulciss* plant extract has highest antioxidant activity close to the standard ascorbic acid (positive control). So these plants are the significant source of natural antioxidant that can be used in cosmetic, food supplement, drug designing etc. From the phytochemical analysis, we found that each of these plant species possesses pharmacologically active biomolecules which may be the attribute of their folk medicinal use. From the result of preliminary cytotoxicity testing (Brine-shrimp lethality assay), only *P. fascicularis* extracts found to be the cytotoxic LD50 value of *P. fascicularis* root extract was found 25.64 µg/ml. Further biochemical investigations are in progress for purification and characterization of bioactive molecule present in the above described four medicinal plants.

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PRESENT STATUS OF FISH AVAILABILITY IN RUHUL *BEEL*, NORTHERN BANGLADESH

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Abstract

This study was conducted in Ruhul *Beel*, Pabna, northern area of Bangladesh to know the availability of different fish species. Samples were collected fortnightly from the fishermen catch captured in different points of the *Beel* during January to December, 2013. During this study a total of 37 fish species under 9 orders and 19 families were recorded from the study area. Cypriniformes was the most dominant order constituting 32.43% of the total fish populations followed by the Siluriformes (27.02%), Perciformes (21.62%), Channiformes (5.4%) and Clupeiformes (2.7%). On the contrary, Belontiiformes, Cyprinodontiformes, Osteoglossiformes and Tetraodontiformes were the least numerous orders constituting only 2.71% each of the total fish populations. Among the available fish species, 37.84% were very rare, 35.14% were rare, and 21.61% were found throughout the year in a small amount while only 5.41% were available throughout the year in a large amount. About half of the fish species available in the Ruhul *Beel* is threatened to extinct according to IUCN Bangladesh. The results of this study will provide important baseline data on availability of fish species, which will be helpful for the sustainable management and conservation of fisheries diversity in the Ruhul *Beel* as well as in the open water-bodies of Bangladesh.

Key words: Bangladesh, fish availability, Ruhul *Beel*, threatened fish

Introduction

Freshwater fishes are the most diverse group of vertebrates, constitutes a vital component of the world, with a species richness compared to both terrestrial and marine ecosystems (Gleick 1996, Duncan and Lockwood 2001). Freshwater biodiversity has declined faster than either terrestrial or marine biodiversity over the years (Jenkins 2003). The major conservation problems of freshwater fish is the fact that the freshwater fish occupy comparatively limited space with much reduced ability for inter-basin movement (UNESCO 2003, Stoddard et al. 2006) contrary to the fairly free moving marine fish (Andrew and Pepperell 1992). Freshwater biodiversity is in a state of critically endangered due to the climate change and habitat destruction (Stoddard et al. 2006). In Bangladesh, human activities and annual environmental effects e.g., dry season and flooding continue to cause considerable damage to the production of inland freshwater ecosystems as well as some fishing methods also been criticized as being environmentally damaging (Andrew and Pepperell 1992). The collection of fish fry during pre-monsoon season, heavy siltation during rainy seasons negatively impacts the inland open water fisheries production as well as growth and development of fish species (Mondol et al. 2015). In 2000, a study by IUCN revealed that 54 of the major inland fish species in Bangladesh are threatened at varying degree (IUCN 2000). Among the commonly cited causes for the deterioration of the fishery resources is the loss of fish habitat, largely caused by the construction of roads, embankments, drainage, flood control and occurrences of natural siltation, along with over fishing (Hughes et al. 1994, Ali

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1997). Enactment of the existing legislations is required for initiating sustainable fisheries management program for conserving or developing the resources.

Knowledge of the existing resources and their condition are therefore essential for planning of any management program for conserving or developing the resources. Ruhul *Beel*, is an important freshwater fish habitat in northern Bangladesh. However, due to various anthropogenic and natural reasons water flow of this *beel* decreased significantly in recent years and results in threatening of the aquatic biodiversity. To the best of our knowledge, there is no previous scientific information on the availability of fish species in this *beel*. Therefore, this study was designed to investigate the fish species availability in the Ruhul *Beel*, Pabna, northern area of Bangladesh.

Materials and Methods

The present study was conducted in the Ruhul *Beel* (24°14'N and 89°24'E) at Bhangura upazila under Pabna district of northern area of Bangladesh. The *beel* covers about 100 hectare in area and is one of the prime habitat for fresh water fishes in Bangladesh. Fish samples were collected fortnightly from the fishermen catch captured in different points of the *beel* during January to December, 2013. Fish were caught using Bhesal jal (Khara jal), Ber jal, Moi jal, cast net and monofilament fixed fill net. Collected fish samples were fixed in 10% formalin at the site and identification was done upon arrival at the laboratory based on their morphometric and meristic characteristics according to Talwar and Jhingran (1991) and Froese and Pauly (2011). Fish availability, threats to fish diversity and conservation needs were determined because of fish abundance during sampling, market survey, interviewing fishermen and fish cognate personnel using pre-structured questionnaire (Rahman et al. 2012 a,b). Fish availability was classified as TY (throughout the year), TYS (throughout the year in small amount), TYL (throughout the year in large amount), SM (found in small amount during monsoon), LM (found in large amount during monsoon), R (rare), VR (very rare) and NE (not evaluated) (Mondol et al. 2015).

Results and Discussion

The present study recorded a total of 37 fish species under 9 orders and 19 families from the Ruhul *Beel* (Table 1). Cypriniformes was the foremost order constituting 32.43% of the total fish population followed by the Siluriformes (27.02%), Perciformes (21.62%), Channiformes (5.40%) and Clupeiformes (2.70%). On the contrary, Beloniformes, Cyprinodontiformes, Osteoglossiformes and Tetraodontiformes were the least numerous orders constituting only 2.71% each of the total fish population (Fig. 1). The dominance of the orders Cypriniformes, Siluriformes and Perciformes in freshwater fish population is common in Indian subcontinent (IUCN Bangladesh 2000, Shinde et al. 2009, Emmanuel and Modupe 2010, Khan and Hasan 2011, Nyant et al. 2012, Mondol et al. 2015). Cyprinidae was the most dominant family constituting 24.32% of the total fish population.

Among the available fish species, 37.84% were very rare while 35.14% were rare, 21.61% were found throughout the year in a small amount while only 5.41% were available throughout the year in a large amount (Fig. 2). 8.1% of the available species were critically endangered, 13.51% were endangered and 16.21% were vulnerable, 8.1% were data deficient, 51.35% were not threatened, 2.70% were not listed according to IUCN Bangladesh (2000).

Still to date previous studies on fish availability in the Ruhul *Beel* are unavailable in the literature. Therefore, this documentation on species availability will compose the foundation for further comprehensive studies. However, it is imperative that many of the threatened to extinction fish species be still dwelling in the Ruhul *Beel*. Our preliminary survey showed that, abstraction of water for irrigation, use of agro-chemicals in

surrounding crop fields besides the *beel*, over exploitation and indiscriminate use of gears are the main threat for fish species conservation in the Ruhul *Beel*. Besides, there is only one-way management policy is to make sanctuary in the deepest part of the *beel*, where fishing is completely banned. But this policy is not properly followed. The implementation of this sanctuary and some other *beel* management policy like, seed production, MACH (Management of aquatic resources through community husbandry), CBFM (Community based fisheries management), and integrated aquaculture technologies should be adopted to protect the species which are a degree of extinction and to recover sustainable production of the Ruhul *Beel*. For this purpose, the following immediate actions may be recommended such as prohibition on harvesting brood fish, stocking of fish fry and ban of fishing by illegal gears. Therefore, it is most urgent to take suitable step to protect fish fauna of Bangladeshi *beel*. In the light of the present study of Ruhul *Beel*, it is the time to make proper policies and necessary steps to implement so that the future generation can get the fishes lively on the earth rather than photographs in literature.

Table 1. Check-list of fish availability in the Ruhul *Beel*, northern Bangladesh.

Order	Family	Scientific name	Local name	Availability	Present status
Beloniformes	Belonidae	<i>Xenentodon cancila</i>	Kaikka, Kakila	TYS	NT
Channiformes	Channidae	<i>Channa striatus</i>	Shol	R	NT
		<i>Channa punctatus</i>	Taki	TYL, LM	NT
Clupeiformes	Engraulidae	<i>Gudusia chapra</i>	Chapila, Khoira	R	DD
Cypriniformes	Cobitidae	<i>Botia dario</i>	Rani, Bau Mach	VR	EN
		<i>Botia lohachata</i>	Rani, Bau Mach	VR	EN
		<i>Lepidocephalus guntea</i>	Puiya, Gutum	R	NT
	Cyprinidae	<i>Ablypharyngodon mola</i>	Mola, Moa	TYS	NT
		<i>Esomus danricus</i>	Darkina	TYS	DD
		<i>Hypophthalmichthys molitrix</i>	Silver carp	R	NT
		<i>Labeo calbasu</i>	Kalibaus	R	EN
		<i>Labeo rohita</i>	Rui, Rohit	R	NT
		<i>Systomus sarana</i>	Sar-punti	VR	CR
		<i>Puntius sophore</i>	Jat punti, punti	TYS	NT
		<i>Pethia ticto</i>	Tit-punti	TYS	VU
		<i>Salmostoma phulo</i>	Chela	VR	NT
		<i>Aplocheilichthys panchax</i>	Panchax	R	NT
		<i>Notopterus notopterus</i>	Foli, Kanla	VR	VU
Cyprinodontiformes	Cyprinodontidae	<i>Aplocheilichthys panchax</i>	Panchax	R	NT
Osteoglossiformes	Notopteridae	<i>Notopterus notopterus</i>	Foli, Kanla	VR	VU

Table 1 Contd.

Perciformes	Anabantidae	<i>Anabas testudineus</i>	Koi	R, SM	DD
	Osphronemidae	<i>Colisa fasciatus</i>	Kholsha	TYL, LM	NT
	Ambassidae	<i>Chanda nama</i>	Chanda	TYS, SM	VU
	Gobiidae	<i>Glossogobius giuris</i>	Baila, Bele	R	NT
	Mastacembelidae	<i>Macrognathus aculeatus</i>	Tara baim	R	VU
		<i>Mastacembelus armatus</i>	Tara baim	R	EN
		<i>Mastacembelus punctatus</i>	Guchi	TYS	NT
Siluriformes	Nandidae	<i>Nandus nandus</i>	Bheda, Meni	VR	VU
	Bagridae	<i>Mystus cavassius</i>	Kabshi tengra	VR	VU
		<i>Mystus tengara</i>	Tengra	R	NT
		<i>Mystus vittatus</i>	Taila air	R	NT
	Heteropneustidae	<i>Heteropneustes fossilis</i>	Shing	TYS	NT
	Schilbeidae	<i>Clupisoma garua</i>	Ghere, Gharua	VR	CR
		<i>Eutropiichthys vacha</i>	Vacha	VR	CR
		<i>Pseudeutropius atherinoides</i>	Batasi	VR	NT
	Siluridae	<i>Ompok bimaculatus</i>	Kani pabda	VR	EN
		<i>Wallago attu</i>	Boal	VR	NT
	Sisoridae	<i>Gagata gagata</i>	Gun tengra	VR	NT
Tetraodontiformes	Tetraodontidae	<i>Tetraodon cutcutia</i>	Potka	VR	NT

TYL, throughout the year in small amount; TYL, throughout the year in large amount; SM, found in small amount during monsoon; LM, found in large amount during monsoon; R, rare; VR, very rare; Status in the IUCN Red List according to IUCN Bangladesh (2000), EN-Endangered, VU- Vulnerable, CR- Critically endangered DD-Data deficient, NT- Not threatened, NL- Not listed.

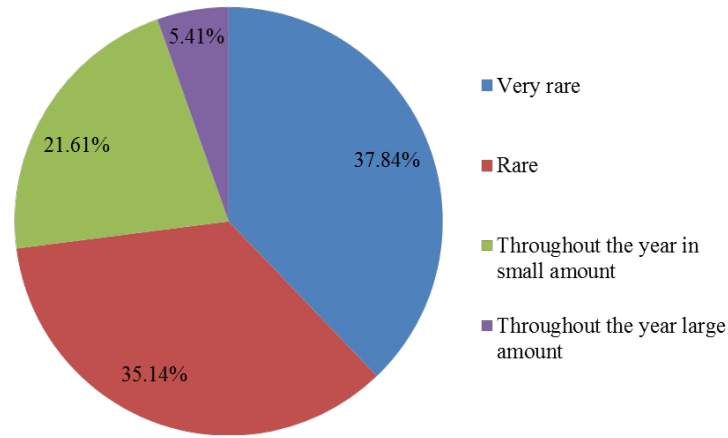


Fig. 1. Fish species availability (percentage) in Ruhul *Beel* during the study period.

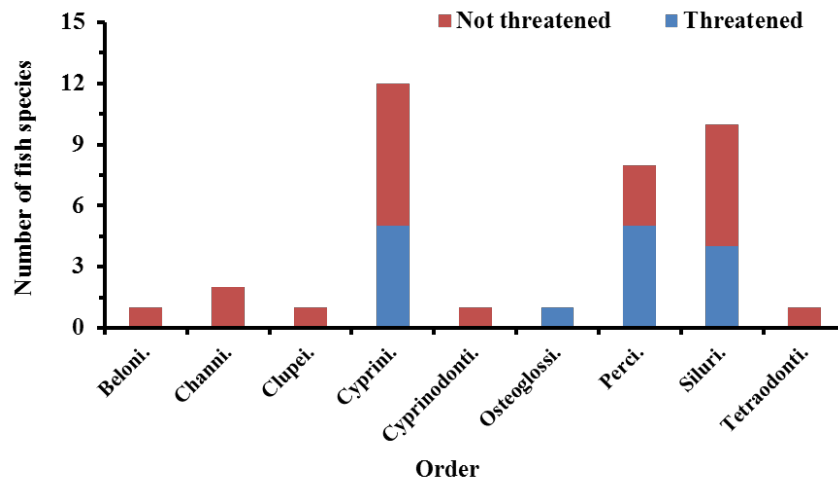


Fig. 2. Number of threatened to extinction and non-threatened fish species under different order in Ruhul *Beel* during the study period.

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BARRIER AND PHYSICAL PROPERTIES OF ARROWROOT STARCH-CARRAGEENAN BASED BIOFILMS

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Abstract

The demand of environmentally friendly packaging materials to overcome the high use of plastic led to increase of research effort on the development of biodegradable plastic from sustainable packaging. In this research, carrageenan films containing of arrowroot starch in the polymer blend were obtained using polyethylene glycol as plasticizer. The objective is to study the effect of different concentrations of arrowroot starch (20%, 40%, 60%, 80% of carrageenan based) on barrier property (water vapor transmission rate), mechanical properties (thickness, tensile strength and elongation at break), solubility in water and whiteness index of the obtained biofilm samples. The result shows that all of them have good transparency, with 60% of concentration gives the highest Whiteness Index. The concentration of arrowroot starch that shows the best resistance to tensile strength is 40%, as well as in thickness values. Concentration of 80% shows the lowest water vapor transmission rate (WVTR) permeability with 85.12 g/m².24h, while the lowest water solubility is obtained from 60% concentration with 60.49% solubility. The recommended level of arrowroot starch to be incorporated in the process of arrowroot starch-carrageenan-based film is 60% of carrageenan.

Key words: Arrowroot starch, carrageenan, film, packaging, polyethylene glycol

Introduction

Over the past 50 years, many packaging materials use synthetic petroleum polymers as their primary source. However, the use of -petroleum-based polymers has been a problem because it has poor biodegradable capabilities (Shojaee-Aliabadi et al. 2013). The use of renewable resources that can produce biodegradable materials to reduce waste disposal problems continues to be explored as consumer concern rise on limited natural resources and environmental issues (Tavassoli-Kafrani et al. 2016). The consumer demand has shifted to eco-friendly biodegradable materials, especially from renewable agriculture by-products, food processing industry wastes and low-cost natural resources (Alves et al. 2006).

Biopolymers are biodegradable, biocompatible, and renewable. Biopolymers can also be eaten in the case of natural biopolymers. Biopolymer packaging materials also have been functioned as gas and solute barriers and can complement other types of packaging by improving quality and extending the shelf life of foods (Rhim 2012). The formation of edible film can be applied by using different biopolymers such as polysaccharides, proteins, and their blends. Polysaccharide films are made from starch, alginate, cellulose ethers, chitosan, carrageenan, or pectin, and exhibit excellent gas barrier properties (Vieira et al. 2011). In recent years, due to their good barrier properties to oxygen, carbon dioxide, and lipids as well as their superb

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mechanical properties (tensile strength and elongation at break), carrageenan and alginate have been used frequently (Tavassoli-Kafrani et al. 2016). Kappa-carrageenan can produce a transparent film with excellent mechanical and structural properties, including a tensile strength higher than those of iota and lambda carrageenan films (Park 1996). However, carrageenan and alginate have high hydrophilic features, so they have limitations as a moisture barrier (Varela and Fiszman 2011).

Another polysaccharide that can be used for source of biodegradable films is starch. One of the natural polymers that can be cast into films is starch. Like other polysaccharide polymers, physical properties of starch films are poor (Abdou and Sorour 2014). Mechanical properties of starch films have disappointedly lagged behind petroleum-based films (Chang et al. 2012). Several compounds are being added to perform a better composite film. Improvement of starch film properties can be made by blending with synthetic polymers to produce biodegradable materials (Lawton and Fanta 1994), mixing with other natural polymers in edible packaging (Arvanitoyannis et al. 1998), or adding plasticizer. Several studies about starch-carrageenan blended films to enhance their film properties have been done (Lafargue et al. 2007, Larotonda 2007, Alves et al. 2010, Moreira et al. 2011, Abdou and Sorour 2014,).

Blending of starch with kappa-carrageenan results in the formation of an edible coating with excellent film forming and mechanical properties (Abdou and Sorour 2014). The addition of carrageenan to starch-based systems results in various physical effects which are now well documented (Larotonda 2007). Carrageenan can be functioned as a starch solution thickener, a gel-accelerating or gel-retarding agent, a gel-strengthening or gel-weakening agent, depending on the polysaccharide type (Lai et al. 1999). However, little is known about the effect of carrageenan on the properties of film cast from carrageenan-arrowroot starch mixtures. A new resource used for making biodegradable films from biopolymers is arrowroot starch. Research about the production of arrowroot starch edible films have been done previously, as a composite with other polymers (Wafiroh et al. 2011) or as an individual source (Yulianti and Ginting 2012). In this experiment, the production of composite arrowroot starch and carrageenan-based films is being investigated.

Materials and Methods

Materials

Kappa carrageenan was obtained from *Eucheuma cottonii* collected in Serang, Banten Province, Indonesia. While, iota carrageenan was extracted from *Eucheuma spinosum* harvested from Nusa Penida, Bali Province, Indonesia. The extraction process of both carrageenan followed the procedures described by Peranginangin et al. (2011). Seaweeds were soaked and then were processed through an alkaline treatment. The extraction process was performed at 60 - 65°C for 2 hours in 8% KOH solutions. The seaweed and KOH solution ratio in the extractor tanks was 1:6 (w/v). Alkali treated seaweed then was rinsed with tap water until it reaches pH neutral (pH 7.0). Second extraction was performed at 80 - 85°C for 2 hours in water with seaweed and water ratio 1: 20 (w/v) by adding 3% of celite. The extract was then filtered using filter press. The concentrated extract was precipitated with Isopropyl alcohol. The precipitate was filtered and then sun dried for couples of days. The dried carrageenan was then turned into powder using a hammer mill. Arrowroot starch was obtained from traditional market in Jakarta, Indonesia.

Blend and film formation

Arrowroot starch was blended with carrageenan by different ratios of starch with concentration 0, 20, 40, 60, and 80% of carrageenan weight (w/w) and mix of kappa and iota carrageenan (1:1) with concentration of 1.5% w/v using glycerol as a plasticizer. The starch and mixed-carrageenan were mixed in distilled water with the total volume of 100 ml. The arrowroot starch and carrageenan mixtures were cast onto flat and leveled glass plates (16 × 16 cm), then the plates were held at room temperature 36-37°C for 24 hours. Afterwards, the films were then peeled off from the glass plates.

Water vapor transmission rate

Water vapor transmission rate (WVTR) of arrowroot starch-carrageenan biofilms was measured gravimetrically using ASTM-E 96/E96 (ASTM 2012b).

Whiteness index measurement

Whiteness index (WI) of the films was assessed through measuring the color using Hunter Lab colorimeter (Coloflex-EZ) to determine the value of L^* , a^* , b^* . The test was performed in accordance with ASTM D2244 (ASTM 2011) using a D65 illuminant with an opening of 14 mm and a 10° standard observer. The colorimeter was calibrated using a standard white plate ($L^* = 93.49$, $a^* = -0.25$, $b^* = 0.09$). The color measurements were performed by placing the film specimens over colorimeter with at least three points of each sample selected randomly to measure the color parameter of films. The following equation was used to calculate Whiteness Index (WI):

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

(Eq. 1)

where L^* , a^* , and b^* are the color value of the arrowroot starch-carrageenan films samples and L , a , and b are the color parameters of the white standard tile.

Mechanical properties

Films specimens were cut into rectangular strips 1 cm wide and 15 cm long after conditioning in 50% relative humidity for more than 70 hours. Tensile strength and elongation at break of arrowroot starch-carrageenan films were determined using STROGRAPH-RI (Toyoseiki, Japan). According to ASTM standard method D882 (ASTM 2012a). The initial grip separation and cross-head speed were set to 50 mm and 50 mm/min, respectively. A microcomputer was used to record the stress-strain curves with a minimum of five replicates of each films tested.

Thickness of the film was determined using Mitutoyo micrometer (made in Japan) with measurement range of 0.01 - 20.00 mm.

Water solubility

The films solubility was determined by a method adapted from Shojaee-Aliabadi et al. (2013). The films samples were cut into square pieces of 0.4 cm² and accurately weighed to record the dried film mass. The films were placed into test beakers with 100 ml distilled water. The samples were immersed and shaken under constant agitation at 180 rpm for 6 hours at 25°C. After that period, the remaining pieces of films were

then filtered and dried in a hot air oven at 110°C until a final content weight was obtained. The percentage of solubility of the films was calculated according to the equation $WS (\%) = ((W_0 - W_f) / W_0) \times 100$, where W_0 is the initial weight of the films expressed as dry matter and W_f is the final weight of the desiccated undissolved films.

Results and Discussion

Seaweed characteristic

The seaweed was characterized prior to use in the process of biofilm production. This characteristic is important to know the quality of the seaweed. The characteristic of seaweed *E. cottonii* and *E. spinosum* is shown in Table 1. It can be seen that all parameters of Indonesian National Standard for dried seaweed number SNI 2690:2015 (BSN 2015) can be met by the dried seaweed. This is indicating that the raw materials used in this process were classified as good quality.

Table 1. Seaweed characteristic for raw materials of refined carrageenan.

Parameter	Indonesia National Standard number SNI 2690:2015*	<i>E. cottonii</i>	<i>E. spinosum</i>
Moisture content (%)	Max 30.0	24.72 ± 1.11	25.85 ± 2.34
Clean anhydrous weed (CAW; %)	Min 50.0	37.14	50.76
Impurities (%)	Max 3.0	0.84	0.83

*source : (BSN 2015)

Characteristic of kappa and iota carrageenan

The dried seaweed was then extracted to obtain the carrageenan using method explained previously. Table 2 shows the physiochemical characteristic of the kappa-carrageenan and iota-carrageenan.

Table 2. Characteristic of Kappa-carrageenan and Iota-carrageenan used in the production of biofilms.

No.	Parameter	Standard		κ-carrageenan	i-carrageenan
		FAO ^a	FCC ^b		
1	Gel strength	Min 5	Min 5	45.00 ± 7.50	47.35 ± 5.50
2	Viscosity (at 75°; cP)	Min 5	-	15.00 ± 2.50	40.00 ± 0.00
3	Moisture content (%)	Max 12	Max 12	22.16 ± 2.29	27.07 ± 0.11
4	Ash content (%)	15-40	Max 35	34.18 ± 1.92	27.15 ± 0.36
5	Acid-insoluble ash content (%)	Max 2	Max 1	0.71 ± 0.15	0.69 ± 0.071
6	Sulphate content (%)	15-40	-	10.45 ± 0.02	2.953 ± 0.23

^a(FAO 2001), ^b(FCC 1981).

The kappa-carrageenan and iota-carrageenan extracted from red seaweed met the standard in most of the parameter set by FAO (2001) and FCC (1981), except for moisture content. This excess moisture may be due to the unfinished drying process. If the carrageenan will be stored for a period of time, this moisture content should be reduced by employing further drying until meet the required level of moisture content.

Properties of arrowroot starch – carrageenan based biofilm

Parameters used to determine the optimum usage level of arrowroot starch on the manufacture of the starch – carrageenan based bioplastics include barrier properties, whiteness index, water solubility and mechanical properties.

Barrier properties

Fig. 1 shows the barrier properties or water vapor transmission rate (WVTR) of the films with various concentration of arrowroot-starch added to the solution of carrageenan. WVTR is important parameter for food packaging due to that the deterioration of foods is affected by moisture transferred from the product's surroundings to the interior products. Fig. 1 shows the lowest WVTR was obtained from 80% concentration of arrowroot-starch (85.12 g/m².24h), while the highest was obtained from 0% concentration with 99.26 g/m².24h of WVTR. It can be seen that WVTR reduced with the increasing of arrowroot-starch concentration. This may be due to that the arrowroot-starch molecule increases the bonds between the carrageenan and reduce the water content of the films, therefore the water vapor transfer through the films also reduce (Nouri and Nafchi 2014). This result is similar with previous study about sago starch film enriched with betel leaves extract (Nouri and Nafchi 2014) and edible cassava starch incorporated with rosemary extract (Piñeros-Hernandez et al. 2017). This is also consistent with previous study by Abdou and Sorour (2014), where the WVTR reduces with the increasing of starch concentration added into carrageenan.

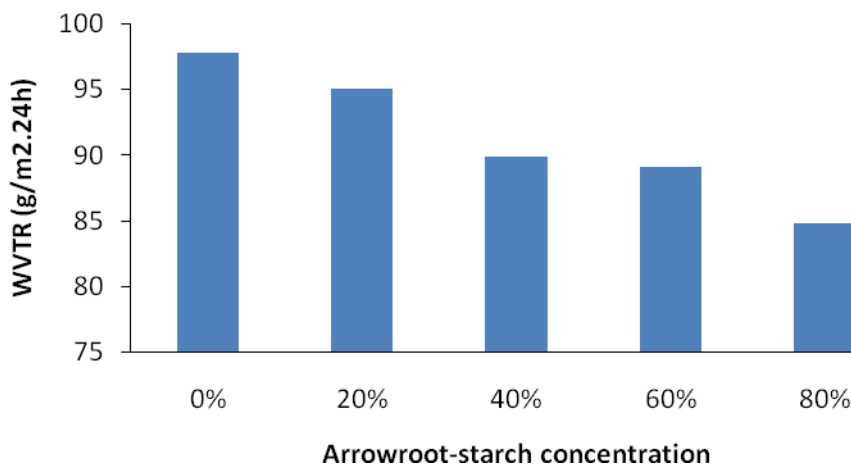


Fig. 1. Water vapor transmission rate (WVTR) of arrowroot starch-carrageenan based films.

Whiteness index

Fig. 2 shows the whiteness index of the films based on colorimetry measurement. The whiteness index was calculated the color parameter values of the film using Equation 1. The film with higher arrowroot-starch concentration gives higher whiteness index. The arrowroot starch content caused the film to have lower transparency meaning that higher arrowroot-starch concentration increases the opacity of the film. Development of transparent packaging materials which allow product visibility is a general trend and requirement in packaging films. Sivarooban et al. (2008) mentioned that the color of the film is an important attribute which influences its appearance, marketability and their suitability for various applications. Clear edible films are typically desirable. Based on the appearance of the films, all of the arrowroot starch concentrations produce biofilm with good transparency. This is important parameter if the films are going to use as transparent food packaging where the appearance of the food can be seen through the bioplastics.

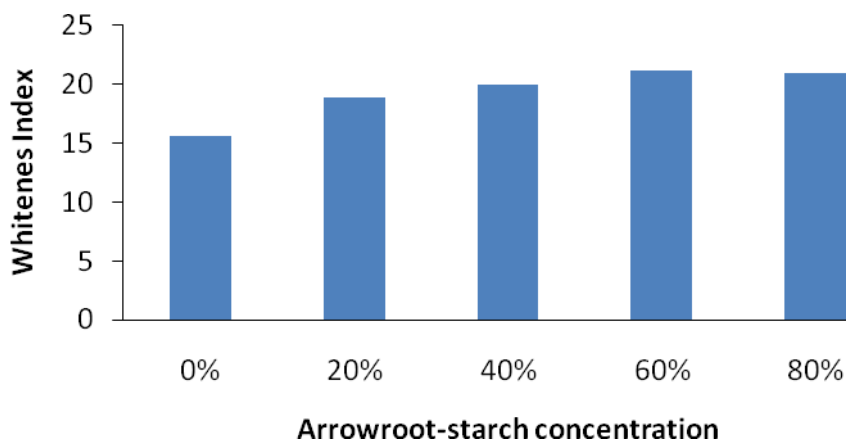


Fig. 2. Whiteness index of arrowroot starch-carrageenan based films.

Water solubility

Fig. 3 shows the water solubility of arrowroot starch-carrageenan based films. The water solubility of the film reduced with the increasing of arrowroot starch concentration, with the lowest obtained from 60% concentration of arrowroot starch (60.49% solubility). However, there was a discrepancy on the value of water solubility on 80% concentration of arrowroot starch, which starts to rise if compared to 60% concentration. It is possibly that there was a maximum concentration of arrowroot starch in the solution which also serves as filler to carrageenan. At 80% concentration the filler become too dense and caused the bonds of carrageenan become weaker. Film solubility in water is an essential property in selecting suitable food packaging plastics. For most food applications, films with good water insolubility are required to provide water resistance and boost shelf-life of food products. However, some food products require packaging films designed to be water-soluble before consumption of the product (Perez-Gago and Krochta 2001). Soluble

film packaging is convenient to use in ready-to-eat products, as they melt in boiling water or in the consumer's mouth (Jirukkakul 2016).

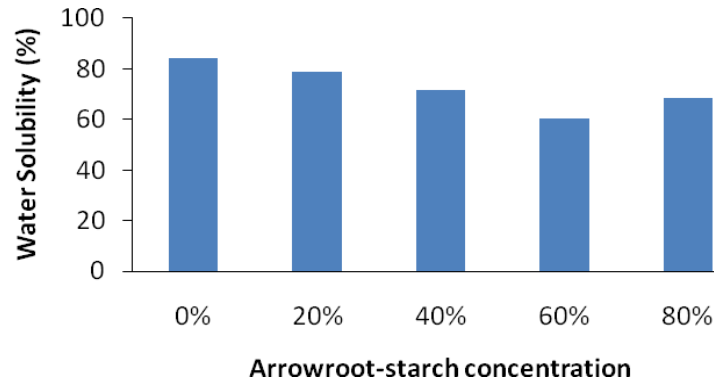


Fig. 3. Water solubility of arrowroot starch-carrageenan based films.

Mechanical properties

Mechanical properties consist of thickness, tensile strength, and elongation at break. The film produced has a plastic-like character due to the characteristics of both arrowroot starch and carrageenan themselves and also by the addition of poly ethylene glycol as a plasticizer.

Thickness

The average thickness of the arrowroot starch-carrageenan based films is shown in Fig. 4. Concentration of arrowroot starch 40% gives the thickest film (0.644 mm), while 0% of concentration gives the thinnest film (0.240 mm). From none to concentration of 40% arrowroot starch shows increasing trends, but after 60% the thickness was reduced. This is possibly because the bonds between carrageenan molecules become weaker for the concentration of arrowroot starch at 60% and above. This value was similar to the reported by Liu and Han (2005) developed pea starch films by extrusion and found thickness values (0.329 to 0.422 mm). Laohakunjit and Noomhorm (2004), developed films of rice starch and glycerol and found thinner values ranging from 0.100 to 0.109 mm being similar to the result informed by Spada et al. (2014) made pinhão starch film with thickness values of 0.11 ± 0.03 mm. Maran et al. (2013) produced very thin film with thickness values ranging from 0.029 to 0.045 mm from film forming solution of starch, glycerol and agar. These differences were suspected probably due to by the different technique and formulation employed to process biofilms.

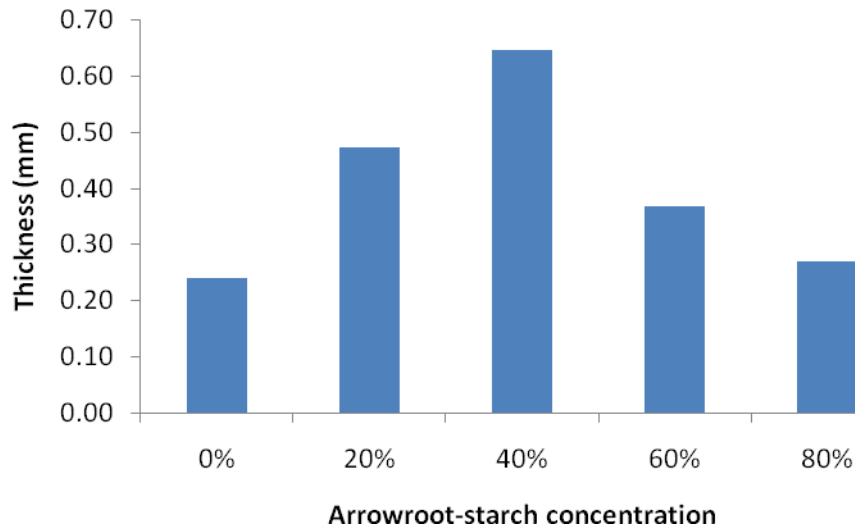


Fig. 4. Thickness of arrowroot starch-carrageenan based films.

Tensile strength

The same trend is also observed from the tensile strength of the films as illustrated in Fig. 5. Arrowroot-starch with 40% concentration gives the best tensile strength (8.292 MPa). This is possibly caused by the excess starch which could loosen the bonds between carrageenan, being consistent with the previous study done by Nazurah and Hanani (2017). Plant oils incorporation to the kappa-carrageenan could make the bonds between polymers become weaker by replacing the bonds with polymer-oil interaction. Ideally the tensile strength should be constant as the film thickness varies, since the material structure is considered homogeneous regardless of the amount of suspension used to form the film. However, on the contrary, the observations showed an important dependence of tensile strength on film thickness. This phenomenon can be explained in terms of the polymer matrix formation during the drying step of the suspension. In the first step, as the water evaporated, a gel structure was formed, and due to further evaporation of water, the first film formed at the interface shrank (Bertuzzi et al. 2012). The high tensile strength values were directly related to the increase in the degree of crystallinity of the polymer matrix (Flores et al. 2007).

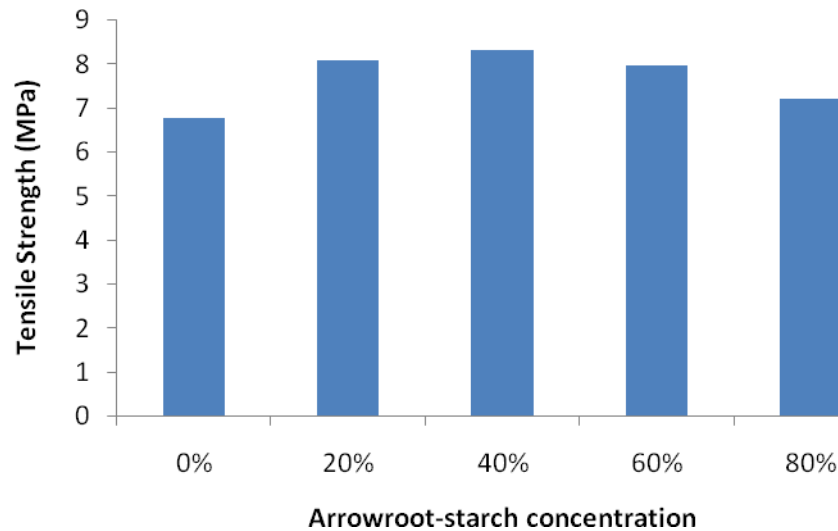


Fig. 5. Tensile Strength of arrowroot starch-carrageenan based films.

Elongation at break

The opposite trend shows on the elongation at break of the films incorporated with arrowroot starch as presented in Fig. 6. A more stretchable matrix was formed in thicker films, probably because these films had a better organization of the starch chains and a greater cross sectional area, permitting greater extension under stress than the thinner films. Jansson and Thuvander (2004) reported the similar occurrences in starch films and Longares et al. (2004) in glycerol-plasticised whey protein isolate films. The best elongation at break is obtained from 20% of concentration, while the lowest elongation occurs from 40% concentration.

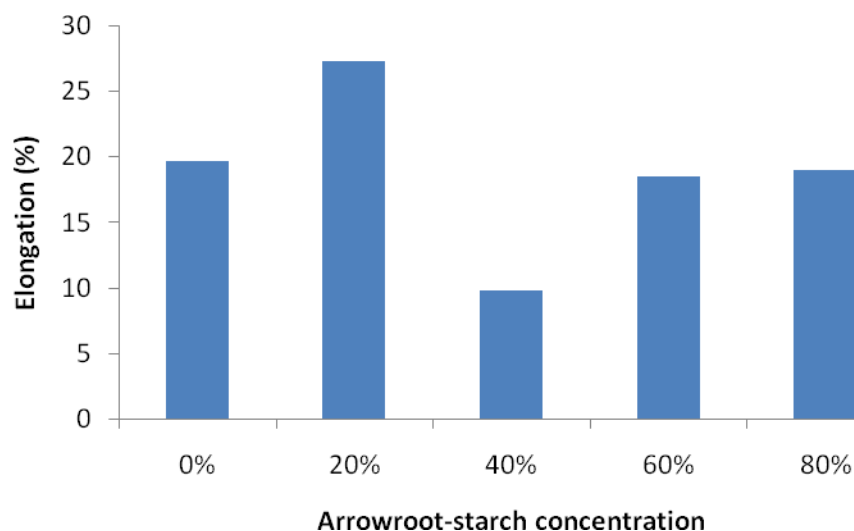


Fig. 6. Elongation at break of arrowroot starch-carrageenan based films.

Conclusion

The results demonstrate that all experimental samples showed transparency; with arrowroot starch concentration 60% of carrageenan gives the highest whiteness index. Concentration of 80% shows the lowest water vapor permeability with 85.12 g/m². 24h of WVTR, while the lowest water solubility is obtained from 60% concentration level with 60.49% solubility. The concentration of arrowroot starch that shows the best resistance to tensile strength is 40%, as well as in thickness values. The addition of arrowroot starch to carrageenan can improve the properties of the films up to 60%, but above that concentration the quality of the films is decreasing. With the variation of the results between 40% up to 80% concentration, a future study with short range of arrowroot-starch concentration need to be done to find a specific value for the manufacturing of biofilm.

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A NOVEL COMPUTATIONAL APPROACH FOR TOXICOGENOMICS BIOMARKER DISCOVERY IN DRUG DEVELOPMENT PIPELINE

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Abstract

In the early stage of drug development process, it is urgent to judge the toxicity effect of some common chemical compounds (CCs) that is not yet well investigated. Biomarker genes (BGs) and dose of CCs can help to draw a deduction about a drug for safety assessment. Classical toxicology method uses large number of samples to extract clinical results which is both time consuming and costly. However, conventional molecular methods can perform to identify only BGs and fail to detect source factor influencing these BGs. The aim of this study is to propose a suitable algorithm that can identify more promising and essential toxicity biomarkers related to some common CCs for safety assessment of new drugs. The *glutathione* is an effective metabolite of detoxification process in liver. *Glutathione* depletion analysis is one of the major key research areas in drug development pipeline. In this paper, we studied *glutathione* depletion analysis of some reported CCs (acetaminophen, methapyrilene and nitrofurazone). We develop an algorithm combining ANOVA and principal component analysis (PCA) using visualization technique to find biomarker genes and associated *glutathione* depleting CCs and their corresponding doses. There are numerous numbers of genes in the *glutathione* metabolism pathway regulated as differentially expressed (DE) genes due to the toxic effect of these CCs and proposed algorithm identify only five genes (*Mgst2*, *Gclc*, *G6pd*, *Gsr* and *Srm*) that are also foremost genes in the *glutathione* metabolism pathway. Proposed algorithm states that high dose of all the CCs are responsible for *glutathione* depletion, nevertheless middle dose of acetaminophen and nitrofurazone also cause *glutathione* depletion. The proposed algorithm has an additional benefit over the conventional method to discover new chemical entities toxicity.

Key words: ANOVA, biomarker, *glutathione*, microarray, PCA, toxicogenomics

Introduction

The chemicals whose have an adverse effect in human body are regarded as toxic agent but this adverse effects are extensively dependent on the chemicals exposure level. For a pharmaceutical drugs, the effectiveness and the toxicity depends on it dose level, overdose may cause toxicity. Moreover, sometimes the dose makes the thing toxic according to an old cliché "the dose makes the poison". These toxic compounds are disposed through four stages like absorption, distribution, metabolism and excretion in a biological system (Davis and Riley et al. 2004, Ekins et al. 2005). The liver is the largest internal and main organ in the human body for metabolism and detoxification of drugs and environmental chemicals (Klaassen et al. 2007). Hepatotoxicity is one of the major causes for withdrawals of some CCs from the market. However, most of the preclinical studies are designed based on liver organ. Classical toxicological research depends on the animal testing to determine the hazard of toxic chemical compound to humans based on well-established cytological, physiologic, metabolic, and morphologic endpoints (Suter et al. 2004) and require a large number of animals and huge time for drawing statistically significant conclusions to assess

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the potential of tumorigenicity in animals and relevant risk in humans (Ulrich and Friend 2002, Waters and Fostel 2004). Therefore, the toxicological testing is of high cost in terms of time, labor, compound synthesis, and animals used. On the other side, toxicogenomics that has attracted widespread attention as an alternative means to study the underlying molecular mechanisms of toxicity and address challenges that are difficult to overcome by conventional toxicology methods (Nuwaysir et al. 1999) as well as gene expression (GE) analysis of animals' target organs after drug administration can help assessing potential toxicity before phenotypic appearance through the use of BGs (Fielden et al. 2007, Uehara et al. 2008). That's why toxicogenomics find its great application in the case of drug development and has become a powerful tool for revealing mechanisms underlying toxicological endpoints and a useful approach for the early detection of potential chemical toxicity (Heinloth et al. 2004, Irwin et al. 2004, Battershill et al. 2005, Searfoss et al. 2005, Kiyosawa et al. 2009).

The Japanese Toxicogenomics Project (TGP) systematically collected chemically-induced GE data from 2002 to 2010 and its first outcome was open Toxicogenomics Project-Genomics Assisted Toxicity Evaluation Systems (TG-GATES). In this study, we use TG-GATES database that stores GE profiles and traditional toxicological data derived from rats (liver, kidney, and primary hepatocytes) and human (primary hepatocytes) cells after administering 170 compounds at the combination of multiple dose and time levels (Igarashi et al. 2015).

In the liver, detoxification process is always continuing and glutathione plays the major role in this process by conjugating target toxic compounds and exports the conjugated compounds into bile ducts. To analyze drug induced glutathione depletion from TG-GATES database, we discover common DE genes for some drugs that are affected by different factors likewise, doses and measurement time levels or combinations thereof. The ultimate goal of toxicogenomics study is to identify genes that are differentially expressed in the conditions (CCs-dose-time) being studied. Comparison statistics, for instance, fold-change, Mann-Whitney U test and t-test are frequently used to discover DE genes in toxicogenomics (Nyström-Persson et al. 2013). Fold-change is the log ratio between two conditions and a threshold value have been used to choose two fold difference whereas Mann-Whitney U test is a nonparametric test also produce same result as like as t-test, a statistical test in normal conditions. The result obtained from fold-change is not so suitable since it does not give any level of confidence to define a DE gene and it's identify wrong gene in case of low intensity with high variance (Newton et al. 2001, Rocke et al. 2001). In toxicogenomic experiment, we have different factors like dose and measurement time points and t-test is compare the expression level of control dose (samples) with the others dose levels by pooling all samples or make many comparisons, one for each different pair of conditions. For toxicogenomics data, t-test is not an efficient approach, because it does not fully utilize all of the information existing in the data and it produces low power because of small sample in each condition (Cui et al. 2003). ANOVA technique considers the variability of the expression levels in different factors. If the variability is present in the expression levels of a gene by the combined effect of dose and time, this indicates that the gene is differentially expressed (Pavlidis et al. 2003). In this study, we consider two-way ANOVA technique that provide interaction effect between dose and time levels of a compound on the expression value of a gene, thereby, the main effects of dose and time levels on that gene are no longer straight forward to interpret (Sahai et al. 2000). PCA can be used to separate dose-time depended cluster of treated CCs group from the control group (Hirode et al. 2008). In this study, combining two-way ANOVA and data visualization based on PCA result, data visualization based on PCA result using common DE genes enables one to identify a group of BGs and to select risk factor (dose of CCs in some toxic mechanisms). Using proposed algorithm, we can easily identify BGs and associated source factors. However, proposed algorithm result permit the histopathological results obtained from the same sample conditions and it is also possible to use proposed algorithm to discover new chemical entities toxicity which is describe in detail in conclusion section.

Materials and Methods

Computational approach

Proposed computational approach to analyses toxicogenomics experiment data are delineated in Fig. 1. Microarray experimental data contain thousands of variables, (i.e. genes) and only relatively few samples, so it is necessary to reduce the number of genes considered for further analysis. This can be done by restricting on a group of genes or by selecting a pathway. However, some compound are influencing a given pathway or biological process of particular interest. For some given compounds, we can reduce the data dimension by selecting a set of genes involving those compound induced pathway. Suppose, we have c compounds data D_1, D_2, \dots, D_c each have same variables or genes (M) and either same or different number of samples depending on dose-time selections of compound. Let us consider the sample size of each compounds are same (m), it seems a balance condition. Again, consider there are n genes are involved in a given pathway. To analyze this pathway, we diminish variables size N to n (after removing others gene from variable list that are not involved in this pathway).

Identification of DE genes

In this study, to identify DE genes for toxicity prediction, we have used two-way ANOVA model. Let us consider Y_{ijk} is a random variable of GE and y_{ijk} is the observed GE value of Y_{ijk} at i^{th} replication of the j^{th} dose of a chemical and k^{th} measurement time level. In two-way ANOVA models, all these variables as varying independently and normally around a mean expression μ_{jk} with a constant variance σ^2 . The two-way ANOVA is therefore given in equation (1).

$$Y_{ijk} = \mu_{jk} + \varepsilon_{ijk} \quad (1)$$

The mean expression value, $\mu_{jk} = \mu + \alpha_j + \beta_k + (\alpha\beta)_{jk}$; where, μ is the grand mean, α_j is the j^{th} dose level effect, β_k is the k^{th} time level effect, $(\alpha\beta)_{jk}$ is the interaction effect of the dose and k^{th} measurement time level and ε_{ijk} is the random error. Now the equation (1) can be written in a vector for a GE measure as

$$Y_{ijk} = \mu + \alpha_j + \beta_k + (\alpha\beta)_{jk} + \varepsilon_{ijk} \quad (2)$$

Where, $i = 1, 2, \dots, n; j = 1, 2, \dots, a; k = 1, 2, \dots, b$. The main objective of the above ANOVA model is to test whether all the main effects (α_j) of the dose levels, the main effects (β_k) of time levels or the interaction effects $(\alpha\beta)_{jk}$ of dose and time levels are statistically significant. Under null hypothesis, for testing the mentioned statement the F statistic are used. On the basis of this statistic, if the null hypothesis becomes rejected (i.e., the main or the interaction effects are significantly different). For each CCs, every gene are tested whether the expression level is changed by the effect of dose and time levels of given CCs. DE genes for each CCs are selected by following the condition of interaction effect p -value < 0.05 .

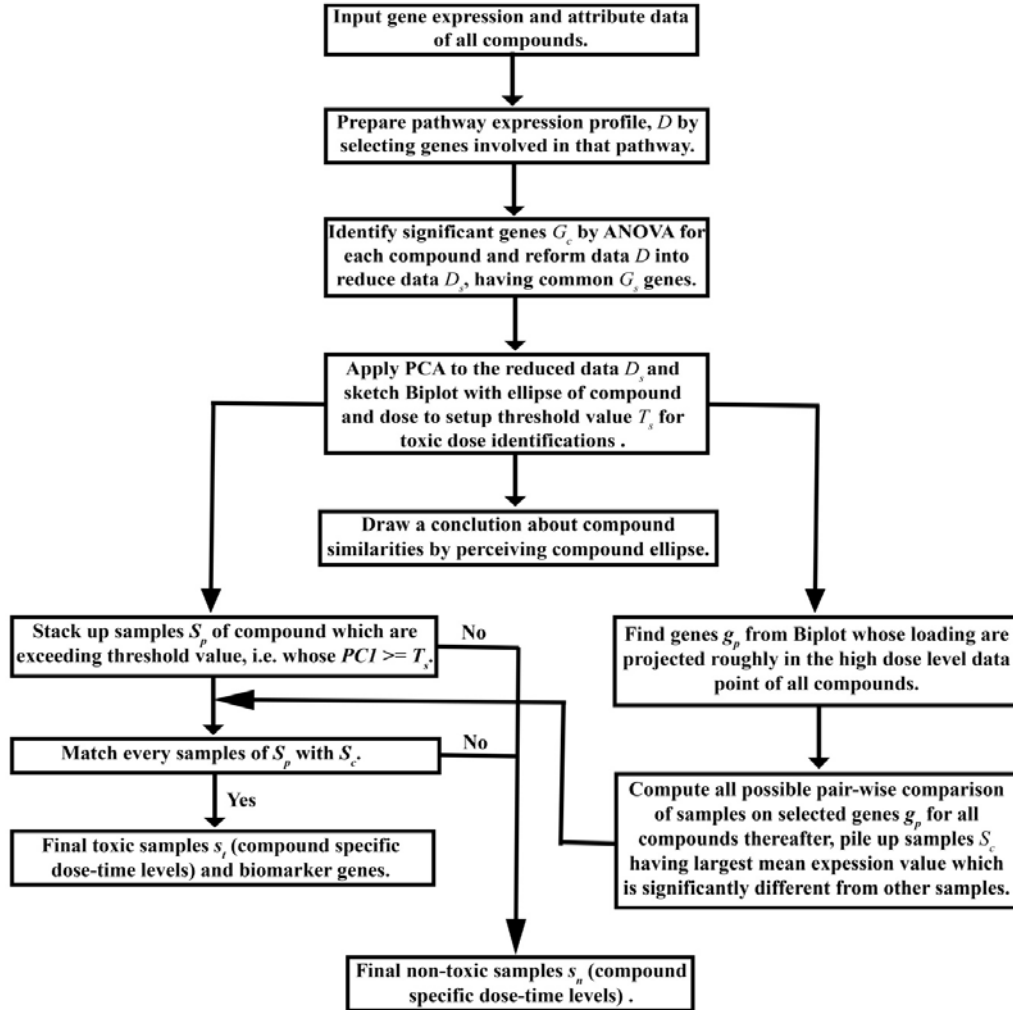


Fig. 1. Overview of the proposed Algorithm.

Identification of BGs and associated source factor

After identifying significant genes from each compound, we extracted common significant genes n_c from the gene list of all CCs which shows much alteration in their expression due to exposure chemicals. Now, apply PCA on the reduce data matrix D_s with dimensions $n_c \times m$. PCA is a promising multivariate technique which is widely used for dimension reduction but in this paper, we used PCA as a classifier or to measure the associations between genes and sample conditions (SCs) i.e., compound specific dose (-time). Combining data visualization and post-hoc testing, multiple comparison tests enables one to differentiate SCs and to find SCs considering expression similarities having highest mean. PCA biplot on significant GE data can be used

to extract the loading sources of variation from toxicogenomics data, to visualize associations between genes and SCs and select BGs from this relationship. We restricts PCA to the first principal component (PC) for selecting probable toxic CCs dose by setting threshold value as smallest absolute loading according to the PC1. It is also possible to make deductions about the groups or similarities of Compound as well as dose by perceiving the scatterings on biplot, similar compound induces GE value might scattered together in the biplot. It is well known that high dose of CCs are more toxic than other dose. Therefore, genes which are projected roughly in the high dose level of CCs are reported as key genes or BGs for toxicogenomic discovery. A gene is DE when it expression patterns shows significant differences in different dose, time or dose-time level. For a specific gene, some doses levels have similar as like control dose and some have different expression value. In the expression level of BGs, SCs are suspected as a toxic compound-dose (-times) whose have high mean expression value. Applying multiple comparisons (Tukey-HSD) post-hoc testing with the condition adjustment of p -value (Holm's), $p\text{-adj.} < 0.05$ to the BGs, we identify putative SCs. Finally, SCs obtained from PCA and post-hoc test are matched and matching SCs are considered as source factor of these DE genes or the desirable compound-dose-time for toxicogenomics discovery. Results from proposed method are tested by the histopathological results to measure the performance of the proposed method.

Source of data

Chemically induced GE data or microarray data refers to toxicogenomics data. According to Nyström-Persson et al. (2013) acetaminophen, methapyrilene and nitrofurazone are the *glutathione* (a major metabolite in detoxification process) depleting compounds. CEL files for microarray GE data after administrating these three CCs were downloaded from TG-GATEs (<http://toxico.nibiohn.go.jp/>). TGP has been taken the scheme collecting high dimensional toxicogenomic data systematically since 2002 as a joint government-privet sector project (Uehara et al. 2010). *In vivo* and *in vitro* are the two main types of data have produced by the TGP, the *in vivo* data, which was collected from *Rattus Norvegicus* at four time points (3, 6, 9 and 24 hrs) for each of four dose levels (control, low, middle, high) from two organs (liver, kidney). In this study, we consider *Rattus Norvegicus's in vivo* liver GE data of chemicals acetaminophen, methapyrilene and nitrofurazone.

Pre-processing and normalization of CEL file

CEL files are mainly image files, prior to computational analysis, it is necessary to quantify image file to expression values. Using the Bioconductor Affy package for R, raw files were pre-processed and mas5 function is used for normalization the GE values.

Results and Discussion

Glutathione depletion analysis

In this study, we have considered three reported glutathione depleting compounds acetaminophen, methapyrilene and nitrofurazone and their toxicity effect under different conditions (dose and time levels) were studied on the 42 probes that are belonging to the *glutathione* metabolism pathway. Examining p -values in the interaction effects between dose and time level, 15, 14 and 17 DE probes out of 42 probes were found for acetaminophen, methapyrilene and nitrofurazone respectively. The p -values of common DE genes in compounds are presented in Table 1. Based on gene ontology, the content of gene related to Glutathione metabolism, glutathione biosynthetic process, glutathione binding, aging, response to cadmium ion, glutathione metabolic process, cytosol were significantly high (Table 2). This feature might reveal that the GE changes in *glutathione* metabolism strongly associated with glutathione depletion or conjugation.

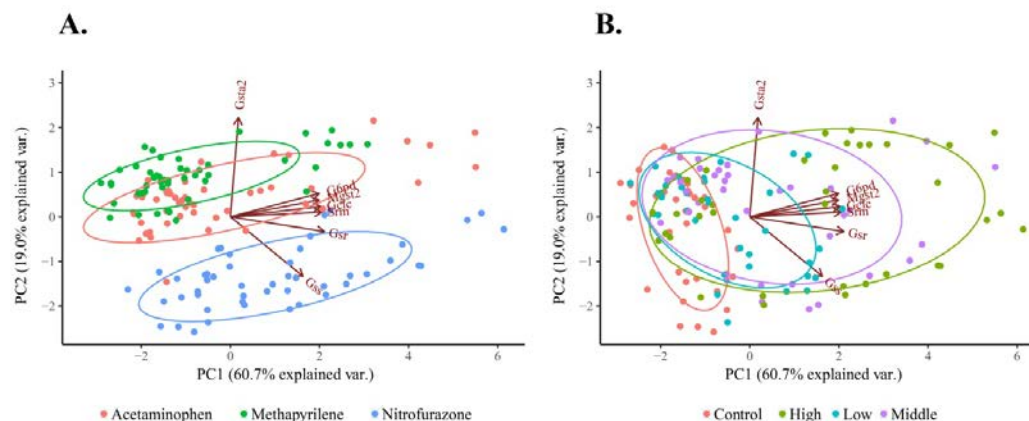


Fig. 2. PCA for common DE genes of all CCs. Biplot of chemical-dose-time data points and common DE genes loading to the first two principal components of the PCA. Different color represents different group. Classification using common DE genes for CCs 'A' (left) and Dose of all chemicals 'B' (right).

Now, we compute PCA on the reduced data matrix based on these seven genes and same SCs. Unless, PCA is generally applied for dimension reduction but in this study, we use PCA for association study. However, the first two components were highly significant contain almost 80% explained variance. The biplot with significant common genes in CCs can be used to correlate SCs and genes. Fig. 2 shows a biplot of SCs and common significant genes with normal ellipse for both CCs and doses. As portrayed in Fig. 2A, acetaminophen and methapyrilene nearly similar compound whereas nitrofurazone is totally different and it's imitate that the mechanism of nitrofurazone may be different from acetaminophen and methapyrilene. From Fig. 2B, we observed that the variation in the data is increases by the increment of doses but doses level don't have any separate cluster. We also observed that most of the low dose data points are scattered with control dose data point and increasing dose data points are scattered far away (especially high dose levels). The biplot enables the association of common DE genes with SCs. The genes, Gsr, Mgst2, Gclc, Srm and G6pd loadings directed according to the first two PCs towards high dose levels of CCs (Fig. 2).

Table 1. Common DE genes for all the CCs in glutathione metabolism pathway.

Gene ID	Gene name	Acetaminophen	Methapyrilene	Nitrofurazone
Gsr	Glutathione reductase	1.91E-07 (4.60E-05)	1.00E-06 (2.30E-05)	8.06E-11 (2.72E-07)
Mgst2	Microsomal glutathione S-transferase 2	1.00E-06 (5.70E-05)	2.00E-06 (0.014261)	3.67E-10 (1.47E-07)
Gclc	Glutamate-cysteine ligase - catalytic subunit	2.50E-05 (0.007903)	1.90E-05 (0.008478)	2.17E-08 (5.00E-06)
Srm	Spermidine synthase-like	0.000408 (0.017384)	4.20E-05 (0.008105)	1.60E-05 (0.993068)
G6pd	Glucose-6-phosphate dehydrogenase	0.001366 (0.026589)	0.000235 (0.22635)	0.000463 (0.700015)
Gss	Glutathione synthetase	0.009570 (0.032616)	0.013921 (0.200432)	0.000617 (0.047296)
Gsta2	Glutathione S-transferase alpha 2	0.017522 (0.006135)	0.023948 (0.341182)	0.001859 (0.906843)

P-values obtained from *t*-test are presented in bracket.

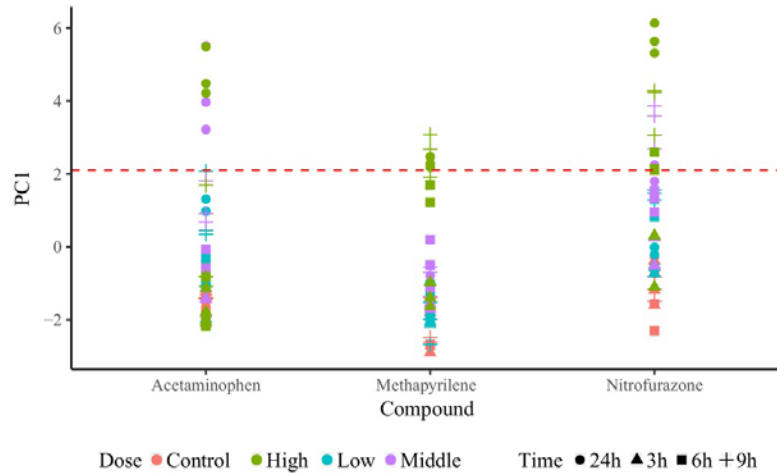


Fig. 3. Score plot for PC1. For each CCs, each data point is duplicated by the combination of different color and shape as shown bottom panel.

The vector of these five genes represent the loadings indicate that they have strong association with the high dose levels (Fig. 2). Therefore, by the proposed algorithm, genes Gsr (glutathione reductase), Mgst2 (microsomal glutathione S-transferase 2), Gclc (glutamate-cysteine ligase), Srm (spermidine synthase) and G6pd (glucose-6-phosphate dehydrogenase) are the biomarker genes for this study as well as glutathione depletion analysis. Gclc is known to accelerate glutathione synthesis and Mgst2 is glutathione-S-transferase, which is the main enzyme for detoxification of toxic compounds by the conjugation reaction. Gsr, G6pd and Srm is a central enzyme of cellular antioxidant defense and reduce oxidized glutathione disulfide (GSSG) to the sulfhydryl form glutathione. The loadings or the vectors indicate that these genes are highly correlated to each other and have strong association with toxic dose. To identify suspected toxic dose of CCs, we setup a threshold value for PC1 score by considering smallest length of these five genes vector. In this study, we chose the threshold value for PC1 score is 2.1 (Fig. 2).

Table 2. Go analysis of identified common seven DE genes.

Term	Count	Percent	P-value
Glutathione metabolism	6	85.7	1.9E-10
Glutathione biosynthetic process	3	42.9	2.4E-4
Glutathione binding	3	42.9	2.5E-4
Aging	4	57.1	2.3E-3
Response to cadmium ion	3	42.9	1.8E-3
Glutathione metabolic process	3	42.9	1.8E-3
Cytosol	5	71.4	4.0E-3

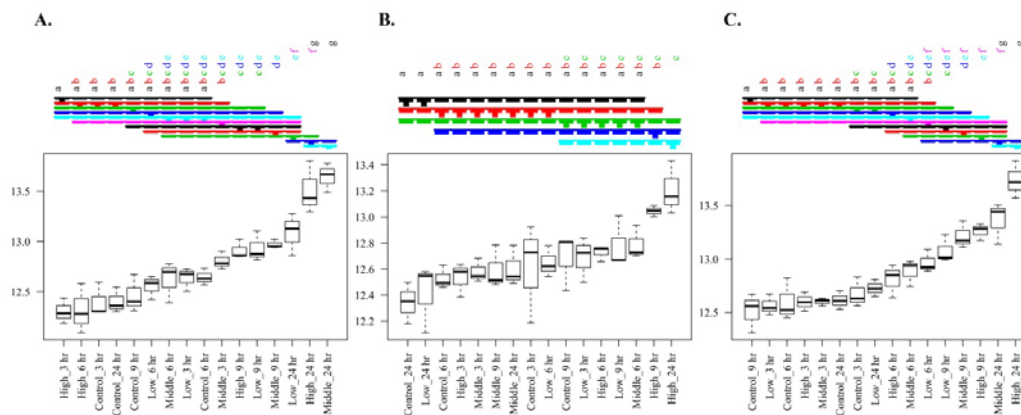


Fig. 4. Boxplot in different SCs for *Mgst2* gene. Lettering in the top panel represent similarity among SCs. Gene expression patterns for only gene *mgst2* in different SCs of acetaminophen 'A', methapyrilene 'B' and nitrofurazone 'C' are displayed.

Only 24 h time point of high and middle doses in acetaminophen, 9 h and 24 h time points of high dose in methapyrilene, and 6 h, 9 h and 24 h of high dose and only 9 h and 24 h of middle dose in nitrofurazone exceeds the threshold value in PC1 score which data points might be responsible for glutathione depletion (Fig. 3). To validate PCA result, we now apply post-hoc testing criterion by the proposed method using these five significant genes. Applying post-hoc test, we found 9 h of low and middle doses, 24 h of high and middle doses in acetaminophen, 6 h, 9 h and 24 h time points of high dose in methapyrilene, and both 9 h and 24 h of high and middle doses in nitrofurazone have comparatively high and similar expression patterns (a boxplot for the expression patterns of gene *Mgst2* from all CCs also shown in Fig. 4).

Table 3. Biomarker genes for all the CCs in glutathione metabolism pathway.

CCs	Dose	Time	Score	<i>Mgst2</i>	<i>Gclc</i>	<i>G6pd</i>	<i>Gsr</i>	<i>Srm</i>
Nitrofurazone	High	24h	5.70	13.74	13.05	12.50	11.81	11.24
Acetaminophen	High	24h	4.73	13.51	13.36	12.47	11.16	10.9
Acetaminophen	Middle	24h	4.23	13.64	13.07	12.04	11.06	10.6
Nitrofurazone	High	9h	3.86	--	13.04	11.54	--	10.91
Nitrofurazone	Middle	9h	3.38	--	13.16	--	--	--
Methapyrilene	High	9h	2.56	13.05	13.38	11.36	10.27	10.81
Methapyrilene	High	24h	2.31	13.21	--	11.76	10.26	10.21
Nitrofurazone	Middle	24h	2.05	13.36	--	--	--	--
Acetaminophen	Middle	9h	1.82	--	12.82	--	--	--
Methapyrilene	High	6h	1.70	--	13.10	10.81	10.16	10.71

-- = not significant (comparatively low expression value than the source factor). Score represent the PC1 score and the value corresponding genes are the mean expression value.

After matching both results from PCA and post-hoc test we found, only high dose of methapyrilene and middle and high doses of acetaminophen and nitrofurazone, which are considered as the responsible source factor for glutathione depletion. The pathological result of these three CCs permits the result obtained from proposed algorithm (<http://toxico.nibio.go.jp/>). We can also rank CCs with their dose levels by PC1 score which may reflect the effectiveness of glutathione depletion. The rank score and significant mean expression of biomarker genes are presented in Table 3.

Conclusion

Biomarker discovery in toxicogenomics research is crucial for assessing adverse effect of chemical in the early stage. Large scale chemical-induced GE data help to study adverse effect of chemicals. Basically, GE changes imitate the pathological condition changes. Therefore, to measure toxic potential of unknown drug by measuring GE fingerprint with some known drug give additional benefits which save both cost and time. From the literature it is known that the compounds, acetaminophen, methapyrilene and nitrofurazone are *glutathione* depleting compound and proposed method identify corresponding dose levels which caused glutathione depletion. Proposed algorithm also identifies five BGs (Mgst2, Gclc, G6pd, Gsr and Srm) that's effectively control glutathione depletion. In mechanistic point of view by the proposed method, nitrofurazone shows slightly different mechanism then the others CCs. It would be possible to identify new chemical toxicity (for instance, *glutathione* depletion) by comparing its expression pattern of BGs with the known CCs. Therefore, further study is obviously needed to rally and simplify the candidacy of BGs suggested in the work.

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METROGLYPH ANALYSIS IN *TRICHOSANTHES DIOICA* (ROXB.)

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Abstract

Morphological variation in eighteen lines of pointed gourd (*Trichosanthes dioica* Roxb.) was determined by Metroglyph method showing their genetic relationship. Assessment of variability of pointed gourd may help for successful utilization of its different attributes in developing suitable genotypes for yield and stability. A total of twelve quantitative characters were used for analyzing genetic variation. Arbitrarily the clusters were found in the diagram but not in consolidated form. Cluster I was represented only by two lines. Almost similarly cluster II was found to be comprised of only three lines. On the contrary, cluster III contains thirteen lines of pointed gourd, although they were found to be located scattered showing their great morphological variations.

Key words: Metroglyph analysis, *Trichosanthes dioica*, variability

Introduction

Pointed gourd commonly called as *potal* in Bangladesh is a dioecious perennial herbaceous vegetable. This crop is of Indo- Malayan origin and distribution, and is extensively grown in eastern India (Chakravarthy 1982) and to a lesser extent in other parts of South Asia (Mythili and Thomas 1999). *Trichosanthes dioica* Roxb. commonly known as "Sespadula" in English and "Parwal" in Hindi, is widely grown throughout India (Shah and Seth 2010). Assam-Bengal region is believed to be the primary centre of origin of pointed gourd (Choudhury 1979). Fruits of this plant are used as vegetable in Indian sub-continental food system. Besides fruits, other parts of the plant, such as the leaves and tender shoots are also used in the traditional system of medicine since ancient times. It is a member of Cucurbitaceae (Chakraborty et al. 1991) and the plant is creeper and grows as vine remains dormant during winter (Nath et al. 1987). Roots are tuberous with long taproot system. Vines are pencil thick in size with dark green cordate simple leaves. Flowers are tubular white with 16 -19 days initiation to anthesis time for pistillate flowers and 10 -14 days for staminate flowers. Stigma remains viable for approximately 14 hours and 40 - 70% of flowers set fruit (Singh 1989).

Traditionally *T. dioica* is multiplied through stem cuttings and root cuttings. Propagation through seeds is not desirable due to poor germination and imbalanced male-female ratio. Seed based populations have a tendency to give more male than female plants and in some cases the ratio goes up to 85:15, limiting their use as their utility ends with pollination (Som et al. 1993).

The assessment of variability present in the crop helps for successful utilization of plant characters in developing suitable varieties for yield and stability. Krishnaprasad and Singh (1991) evaluated twelve genotypes of pointed gourd at the Central Horticulture Experiment Station, Ranchi, India during 1985-86 and 1987-88 growing seasons and found them to exhibit significant difference in all traits. Prasad and Singh

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(1989) found low genotypic and phenotypic variance for node order of first female flower opening (2.68 to 7.42) in ribbed gourd. Arora et al. (1983) found moderate heritability and high genetic advance (69.98 and 48.39) for first female flower opening in sponge gourd.

Anderson (1957) proposed metroglyph and index score method to study the pattern of morphological variation in crop species. This technique has been used by several workers (Ramanujam and Kumar 1964, Mukherjee et al. 1971, Venkatarao et al. 1973, Singh and Chowdhury 1974, Kabir et al. 1993) in various crops. This effort has not yet been carried out in *Trichosanthes dioica*, which may also be of help in predicting their relationship. Thus, a metroglyph analysis was made in different lines of *Trichosanthes dioica* to draw a conclusion for the aforesaid problems.

Eighteen lines of *Trichosanthes dioica* (Roxb.) were used as experimental material in the present study. The vines of the lines were grown under uniform conditions with three replications of each. The seventeen lines were collected from BARI (Ishwardi) and only one from Rajshahi. Observations were made on 5 plants randomly selected for twelve morphological characters of stem, leaf, flower and fruit.

Following metroglyph method, a mean table from the recorded data was prepared where each value was the mean over replications (Anderson 1957, Mehra and Anderson 1969, Singh and Chowdhury 1979). A particular line was represented by a glyph, the X-axis being the stem length and the Y-axis being the leaf area. Ten other characters were represented by rays on glyph, the ray for same character having the same position in each glyph. The range of variation in each character was represented by varying length of rays, i.e., a line having low value with no ray, medium value with short ray and high value with long ray. The index values were decided on the basis of range of variability and were divided into three classes, i.e., 1-no ray, 2-short ray and 3-long ray (Table 1). The total index values were recorded by summing up the index scores of all the 12 characters studied.

The results of morphological analysis (Fig. 1a,b) were considered to study the pattern of morphological variation. The minimum and maximum scores were $n \times 1$ and $n \times 3$, respectively where 'n' was the total number of characters included in the study. The performance of a genotype is denoted by the index score of that genotype and depending upon the score the length of ray varies. Two most variable characters are used for determining the X and Y axis to plot the graph and thus, for construction of metroglyph pattern (Singh and Chaudhury 1979). Stem length and leaf area were the two variable characters in the present study. A keen examination of placement of glyphs on the graph indicated that these two characters were not related to each other. Four characters viz. fruit length, circumference of fruit, weight of fruit and no. of seeds per fruit were recorded from female plants logically. This might have reason for marked differentiation of all the lines studied in this experiment.

In this study the cluster III definitely had high values for those characters which directly contribute to the length of inter-node, no. of inter-node, fresh weight of leaf, days to flowering, length of flower, fresh weight of flower, length of fruit and weight of fruit. It has been observed that the maximum no. of lines i.e. thirteen belongs to the cluster III out of eighteen. There were most similarities, observed between PG19 and PG28 by showing the ray patterns. PG19 showed the maximum Y-value including other variable characters. This

variety also expressed all variable characters by showing short ray on the graph. Cluster II, consisted of three lines. BARI2 and PG06 were found to be correlated only according to X and Y-values.

Table 1. Class intervals, index values and distribution of scores of *Trichosanthes dioica* under different intensities.

Characters	Range of means	Index values					
		1		2		3	
		Range	Sign	Range	Sign	Range	Sign
Length of stem (cm) (X-axis)	68.38-122.81	89.36 (9)		89.37-110.34 (7)		110.35 (2)	
Length of inter-node (cm)	4.47-6.81	5.52 (9)	○	5.53-6.57 (8)	○	6.58 (1)	○
Number of inter-node	12.27-21.11	15.22 (11)	○	15.23-18.17 (6)	○	18.18 (1)	○
Leaf area (cm ²) (Y-axis)	27.13-95.05	59.30 (8)		59.31-91.47 (9)	○	91.48 (1)	○
Fresh weight of leaf (gm)	0.897-3.396	2.06 (9)	○	2.07-3.22 (8)	○	3.23 (1)	○
Days to flowering (first time)	105.06-133.91	118.83 (9)	○	118.84-132.60 (7)	○	132.61 (2)	○
Length of flower (cm)	6.45-8.85	7.30 (10)	○	7.31-8.15 (6)	○	8.16 (2)	○
Fresh weight of flower (gm)	0.695-1.043	0.856 (10)	○	0.857-1.00 (6)	○	1.01 (2)	○
Length of fruit (cm)	6.01-11.50	8.73 (10)	○	8.74-11.45 (6)	○	11.46 (1)	○
Circumference of fruit (cm)	7.09-13.48	10.79 (8)	○	10.80-14.49 (9)	○	14.50 (0)	○
Weight of fruit (gm)	10.55-62.48	37.18 (9)	○	37.19-63.81 (8)	○	63.82 (0)	○
No. of seeds per fruit	16.79-33.29	23.25 (9)	○	23.26-29.71 (7)		29.72 (1)	○

● Number of scores shown in parenthesis

Cluster I was represented by only two lines namely PG08 and PG23. All the minimum variable values were shown by PG23. This line did not show any ray of variable characters. This is further said since PG08 and PG23 are obtained by Cluster I, but there is far difference among other characters between the two lines. Comparatively medium values of characters i.e. length of inter-node, fresh weight of leaf, length, circumference and weight of fruit and highest value of the No. of inter-nodes of BARI1 indicated that it had probably undergone recombination in the part with other lines of the same cluster. This variety showed the highest value of stem length.

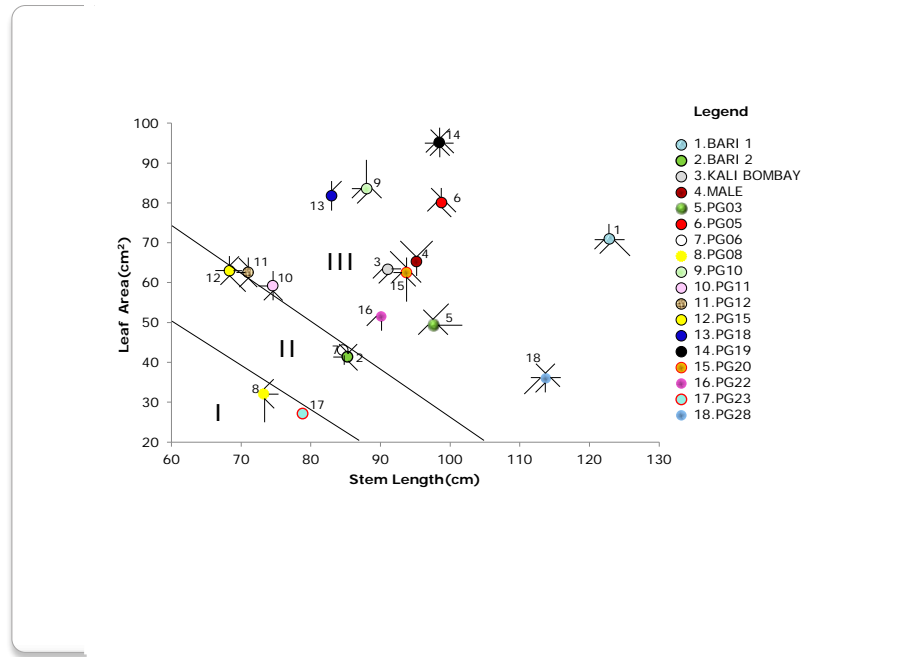


Fig.1a. Metroglyph diagram of various characters in eighteen lines of *Trichosanthes dioica*.

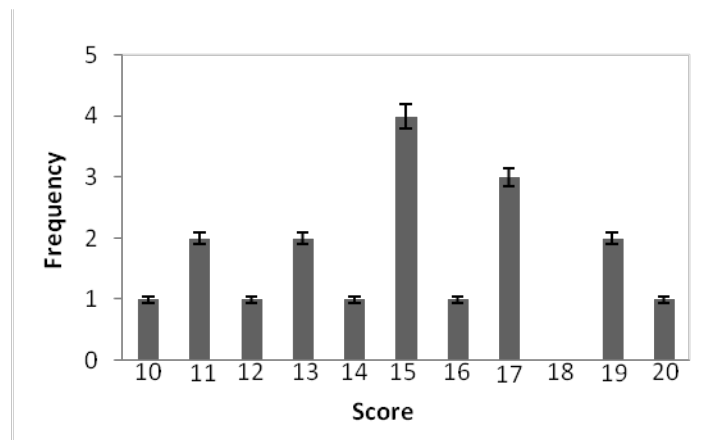


Fig.1b. Index score of eighteen lines of *Trichosanthes dioica*.

Conclusion

From the study it can be concluded that most of the lines of pointed gourd are much differentiated from each other and this is why most of them were found to be scattered on metroglyph graph. It also can be said that most of the characters were obtained having medium values. The present findings also indicated that the genotypes could show less genetic divergence if the number of studied materials could be increased. Moreover, few characters recorded from female plants might have shown distinctive differences morphologically among the lines and that may be reason also for not forming consolidated clusters of the genotypes.

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ANTIBACTERIAL ACTIVITY OF *SYNSEPALUM DULCIFICUM* LEAF EXTRACT AGAINST *LISTERIA MONOCYTOGENES* AND ITS COMPARISON WITH *STROBILANTHES CRISPUS* AND *MORUS ALBA*

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Traditionally, the aqueous extract from *Strobilanthes crispus* has been used by local folks in South East Asian countries including Malaysia and Indonesia for medicinal practices to manage various ailments (Fadzelly et al. 2006). Ethanolic extract of *S. crispus* was found to contain active compounds with good antibacterial activity (Lim et al. 2015). Mulberry (*Morus alba*), a shrub plant from temperate areas with bright red fruit has been used for animal feed and food for human consumption. Since *M. alba* is rich with antioxidant, it has been used widely in cosmetic and drug development (Emniyet et al. 2015). *Synsepalum dulcificum* is a tropical plant which native to West Africa (Chen et al. 2015) and commonly known as miracle fruit due to the unique properties of altering human taste bud by replacing the sour into sweet taste (Njoku et al. 2015). Previously, Chen et al. (2015) reported some effects of miracle fruit leaf extract on mutation and oxidative damage. To date based on our current knowledge; no specific research has been carried out regarding the antibacterial activity of *S. dulcificum* extract. Since *L. monocytogenes* has been reported as resistant to many types of antibacterial drug, finding antibacterial activity compound against it is very difficult (Abbasiliasi et al. 2014). Thus, this research was conducted to investigate the potential of the above mentioned leaf extracts on the antibacterial activity against *L. monocytogenes*.

L. monocytogenes was cultured on brain heart broth (BHB) and incubated at 37°C for two days. After activation, the bacterial was inoculated into BHB and after overnight grown, *L. monocytogenes* was spread onto BHB agar plate. The well (6 mm in diameter) was made using sterile yellow tip. Sample was put in the well and stored in chill temperature (4°C) for 2 hours before incubation (37°C) overnight and the diameter of the clear zone produced was measured (as inhibition zone). The well without inhibition zone was considered as no antibacterial activity. Bacteriocin-like Inhibitory Substance (BLIS) and sterile distilled water (or DMSO-dimethyl sulfoxide) was used as positive and negative control, respectively (Niratker et al. 2015). The DMSO solution was used as received. Antibacterial activity was defined as Activity Unit, AU (mm²/ ml) and the inhibition zone was observed 24 hours after incubation (Abbasiliasi et al. 2014). The calculation of antibacterial activity was performed using the following equation; $AU = (Lz - Ls)/V$. *Lz* represents the clear zone in mm² whereas the *Ls* represents the area of well in mm², divided by the volume of sample in ml

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loaded into the well. Results of antibacterial activity of the both alcoholic extracts were compared with positive control (BLIS) and negative control (DMSO).

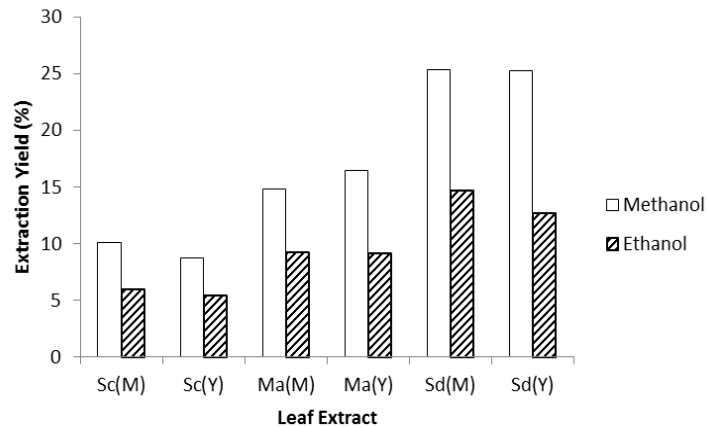


Fig. 1. Yield (%) of methanolic and ethanolic leaf extracts; Sc (M) = *Strobilanthes crispus* (mature), Sc (Y) = *Strobilanthes crispus* (young), Ma (M) = *Morus alba* (mature), Ma (Y) = *Morus alba* (young), Sd (M) = *Synsepalum dulcificum* (mature), Sd (Y) = *Synsepalum dulcificum* (young); Values of each curve are means \pm standard deviation (number of samples, n = 3), P < 0.05.

Fig. 1 shows percentage yield of the methanolic and ethanolic extracts of *S. crispus*, *M. alba* and *S. dulcificum* respectively. Based on the methanolic extracts, the highest yield was obtained from *S. dulcificum* mature leaves of 25.35% followed by *S. dulcificum* young leaves of 25.26% and finally *M. alba* young leaves of 14.78%. Meanwhile, for ethanolic extracts, the highest yield was obtained from *S. dulcificum* mature leaves of 14.66% which was much lower compared to the methanolic extracts.

Table 1. Antibacterial activity of *Strobilanthes crispus*, *Morus alba*, and *Synsepalum dulcificum* extracts on the growth of *Listeria monocytogenes*.

	Activity unit, AU (mm ² /ml)	
	Methanolic extract	Ethanolic extract
Mature (adult)		
<i>S. crispus</i>	-	-
<i>M. Alba</i>	848.34	756.04
<i>S. dulcificum</i>	284.74	271.47
Young		
<i>S. crispus</i>	-	-
<i>M. Alba</i>	583.23	159.06
<i>S. dulcificum</i>	159.06	232.59

Key: (-) = without antimicrobial activity; values are average of three samples of each leaves, analyzed individually in triplicate.

The effect of methanolic and ethanolic extracts (for both young and mature leaves) on the growth of pathogenic bacteria (*L. monocytogenes*) was studied (Table 1). Higher antibacterial activity (against *L. monocytogenes*) for the methanolic extract of *M. alba* was obtained compared to the ethanolic extract. Antibacterial activity of the methanolic extract of *M. alba* mature leaves was observed to be higher than the young leaves suggesting that the mature leaf contains a higher amount of antibacterial compounds against *L. monocytogenes*. Meanwhile, the antibacterial activity against *L. monocytogenes* observed for *S. dulcificum* was smaller than *M. alba*, regardless of the age of the leaves nor the solvents used for the extraction. *L. monocytogenes* was found to be sensitive towards leaf extracts using in this study, even though it is well known as multi-resistance to many drugs (Kümmerer 2009).

Based on the results, it can be concluded that antibacterial compound against *L. monocytogenes* can be obtained from the crude extract of *S. dulcificum* and *M. alba* from both mature and young leaves with better performance from mature leaf of the methanolic extract of *M. alba*. More researches need to be conducted since both extracts shows good result for *L. monocytogenes* and may also good potential for other pathogenic bacteria.

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ANTIFEEDANT EFFECT OF WATER-SOLUBLE ANTHRAQUINONE PIGMENT OF *KERRIA LACCA*

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Many hemipteran insects are phytosuccivorous in nature and predominantly feed on phloem sap, xylem sap or mesophyll cell (Novotny and Wilson 1997, Douglas 2006). Indian lac insects, *Kerria lacca* (Kerr) (Hemiptera: Coccoidea: Tachardiidae (=Kerriidae)) feed on sugar-rich phloem sap (Ahmad et al. 2012) – a chief source of carbon and nitrogen – and excrete excess sugar in the form of honey dew from anal tubercle which becomes a major cause of fungal contamination. Sugar-rich honey dew attracts ants to feed upon them and thus, this ant-hemipteran mutualistic interaction prevents losses by fungus infection (Sharma and Jaiswal 2011). According to Lu et al. (2012), the presence of lac plantation with lac hosting affects the diversity and abundance of ground dwelling ant species. Among the various ant species which attend lac insects, most are harmless to lac culture, this may be due to the feeding deterrence exhibited by the anthraquinone pigments found as body colouration of these insects. Wild type insect are crimson in colour due to the presence of a complex of closely resembling water-soluble polyhydroxy-anthraquinones (laccaic acid A, B, C, D and E), collectively called as lac dye. Yellow mutants possess only the precursor molecule laccaic acid D (Shamim et al. 2016a). Apart from these, certain alcohol-soluble anthraquinones such as desoxyerythrolaccin, erythrolaccin and isoerythrolaccin are also found in the resinous secretion of lac insects, giving it yellowish colouration (Fig. 1).

Available literature suggests that carminic acid, an anthraquinone compound obtained from body pigments of a scale insect *Dactylopius confusus* exhibit antifeedant activity against ants along with various other biological and pharmacological activities and is said to be evolved as a chemical weapon against predation (Eisner et al. 1980). Based on the structural similarities between the anthraquinone present in carminic acid and anthraquinones present in laccaic acids (produced by *Kerria lacca*); it was assumed that lac dye may also exhibit similar effect. This study aimed for assessment of antifeedant activity of the constituents of lac dye i.e. water-soluble polyhydroxy anthraquinones against ants.

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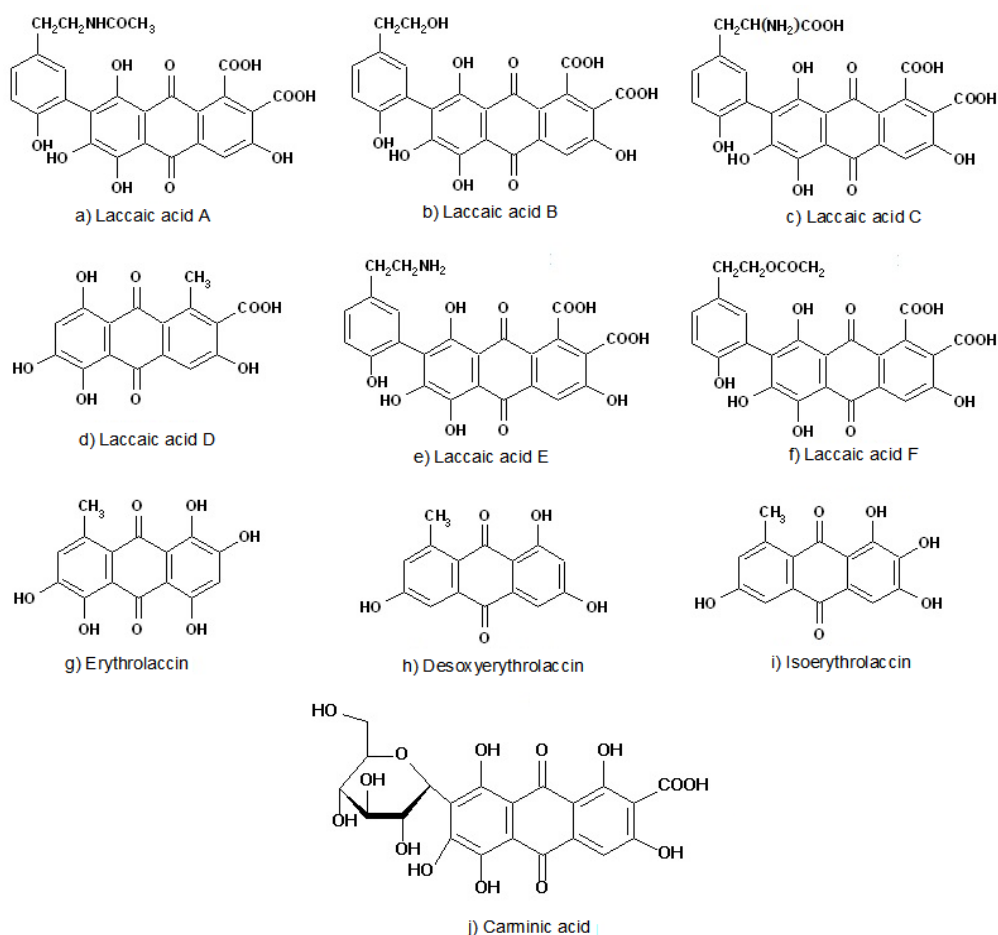


Fig. 1. Chemical structures of polyhydroxy anthraquinone pigments; a - f: Laccaic acids present in lac dye; g - i: laccaic acids found in lac resin; j: Carminic acid produced by *D. confuses* (Shamim et al. 2016a).

For the assessment of antifeedant activity of the anthraquinones found in lac pigments, crimson and yellow insect cell extracts were used along with pure crimson dye, which served as a negative control. This work was carried out at Indian Institute of Natural Resins and Gums (IINRG), Namkum, Ranchi, India and both wild type crimson insects and yellow mutants were obtained from the Field Gene Bank at National Lac Insect Germplasm Centre (NATLIGEC) of the same institution. Also, the crude crimson lac dye was obtained from IINRG's pilot plant facility for lac dye extraction. To purify crude dye, it was dissolved in water and filtered repeatedly using filter paper of 0.19 mm thickness (sartorius stedius biotech, 292a) and air dried at 40°C for 24-48 hrs. Pure dye thus obtained was checked for complete solubility in deionized water. Crude pigment extract of both wild type crimson and mutant yellow insect was prepared simply by grinding the insects in sterile water.

Deterrence of anthraquinones was assessed by offering sugar solutions laced with insect extracts to predaceous ants (Eisner and Meinwald 1965, Carrel and Eisner 1974, Montllor et al. 1991, Hare and Eisner 1993, Schaffner et al. 1994, Morton and Vencel 1998, Gomez et al. 1999, Vencel et al. 1999). Eighty percent sucrose solution was used as a positive control in this study. Sterilized sponges (3.5×1 cm) were soaked with the dye and cell extracts respectively, and then covered with the sucrose solution. One set of pure lac dye and insect extract samples were kept without lacing it with sugar solution. These sponges were kept on sterile slides and were placed near ant hill to assess the antifeedant activity of the anthraquinones under natural conditions where lac insects are being cultured on *Flemingia semialata* plants and are being ant-attended on regular basis. For the similar study under laboratory conditions, the ants were collected from the field where lac insects are cultivated; these ants were left for feeding on the samples under a glass chamber with proper aeration. Feeding behaviour of ants towards the lac dye was observed for an hour and number of ants feeding on it was recorded.

This behavioural study to investigate deterrence of lac dye components led to the observation that yellow dye containing laccaic acid D was most susceptible to ants attack as observed by the maximum number of ants feeding on the sucrose sample overlaid on the cell extract of yellow insects. At the outset of the experiment, it was noted that ants were attracted towards the positive control of experimental set up. They started feeding on the positive control containing only sugar solution but in the test samples i.e. the lac dye coated with sucrose solution, ants visited the periphery and left without feeding. Gradually, the deterrence of the test samples decreased and ants also attended the test samples along with the positive control. No significant result was observed in the negative controls where no sugar solution was added. Pure crimson dye along with sucrose was more attended by ants as compared to the crimson cell extract. At both the assay conditions similar observations were recorded and no significant differences were found (Fig. 2).

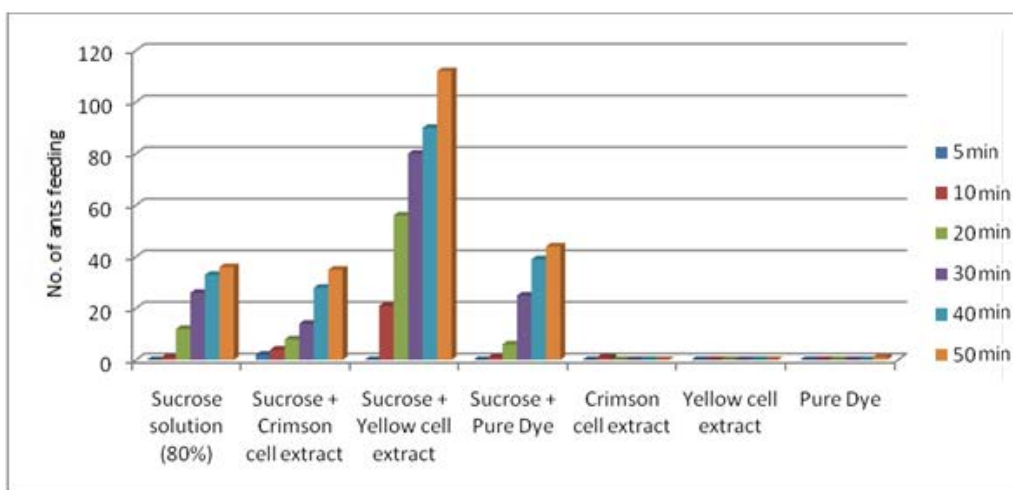


Fig. 2. Graph depicting the observation of the antifeedant assay; yellow cell extract is least deterrent to ants whereas crimson cell extract shows maximum deterrence.

Anthraquinone pigments in insects have been observed to play wide variety of important roles. Apart from being associated with immunity, life-history, physiological, and developmental traits, these pigment molecules exhibit antimicrobial (Cudlin et al. 1976, Manojlovic et al. 2000, Izhaki 2002, Kambizi et al. 2004) and antiviral activity (Barnard et al. 1992, Semple et al. 2001) and protects insects from microbial attack and

viral infections, respectively. For example, carminic acid is reported to show cytostatical activity (Gálvez et al. 1996). Lac dye has also been reported to show antineoplastic effect against leukemia cell lines (Shamim et al. 2016b). Feeding deterrence by anthraquinones is also well reported; anthraquinone synthesised by *Dactylopius confusus* is deterrent against ants (Eisner et al. 1980). Apart from these, several anthraquinones and anthrones showing antifeedant activity against avian predators (Schafer et al. 1983, Hilker and Köpf, 1995, Avery et al. 1997) have found their application in wildlife management, where seeds are treated with anthraquinones against avian pests (Avery et al. 1997).

In this study dealing with antifeedant effect of water-soluble anthraquinone pigment, it was observed that yellow dye containing laccaic acid D was most susceptible to ants attack whereas, a mixture of laccaic acids in crimson dye is more effective in exhibiting feeding deterrence on ants. The pure crimson dye is less effective than the cell extract of crimson coloured insects, it could be due to the loss of deterrence during the processing and purification of the dye or feeding deterrence could be due to a cumulative effect of a mixture of compounds along with the pigment molecule. There is still a gap in the understanding of antifeedant effect of anthraquinones and therefore, it is suggested that *K. lacca* anthraquinones should be explored more as potential natural antifeedant, based on its active constituents.

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