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Research Article

In vitro Antidiabetic Activity of *Sargassum hystrix* and *Eucheuma denticulatum* from Yogyakarta Beach of Indonesia

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Abstract: Marine algae are a potential bioactive source that began to be developed as a new pharmaceutical agent, including antidiabetic. The objective of this research was to determine the potential of polyphenols and phlorotannins extract from *Sargassum hystrix* (J. Agardh, 1847) and *Eucheuma denticulatum* [(N. L. Burman) F. S. Collins & Hervey, 1917] in inhibiting *α*-amylase and *α*-glucosidase. Polyphenols were extracted using 50 % methanol, and phlorotannins were extracted using methanol, and the non-lipid layer was separated by using distilled water, methanol and chloroform, and then partitioned using ethyl acetate twice. The total content of polyphenols and phlorotannins were analyzed. Both types of the compounds were tested to determine their ability to inhibit *α*-amylase and *α*-glucosidase activity. Total phenols content of *S. hystrix* and *E. denticulatum* were observed to be 3.17 g GAE. 100 g^{-1} extract and 0.33 g GAE. 100 g^{-1} extract, respectively. Total phlorotannin content of *S. hystrix* and *E. denticulatum* were obtained 0.02 g PGE. 100 g^{-1} extract and 0.02 g PGE. 100 g^{-1} extract, respectively. The results showed that polyphenols *S. hystrix* (IC_{50} = 0.58±0.01 mg.mL^{-1}) can inhibit *α*-amylase, similar to acarbose (IC_{50} = 0.53±0.00 mg.mL^{-1}) and phloroglucinol (IC_{50} = 0.56±0.01 mg·mL^{-1}), but inhibiton activity of polyphenol and phlorotannin from *E. denticulatum* was lower (IC_{50} = 1.43±0.19 and 1.92±0.14 mg.mL^{-1}, respectively). Inhibitory activity of polyphenols from *S. hystrix* (IC_{50} = 0.59±0.02 mg.mL^{-1}) in inhibiting *α*-glucosidase was also similar to acarbose (IC_{50} = 0.61±0.01 mg.mL^{-1}) and phloroglucinol (IC_{50} = 0.56±0.05 mg.mL^{-1}), but inhibiton activity of polyphenol and phlorotannin from *E. denticulatum* was also lower (IC_{50} = 1.43±0.19 and 0.86±0.06 mg. mL^{-1}, respectively). So, *S. hystrix* had more potential as an antidiabetic substance compared to *E. denticulatum*.

Keywords: *α*-amylase, *α*-glucosidase, Antidiabetic activity, *Eucheuma denticulatum*, *Sargassum hystrix*

1. INTRODUCTION

Diabetes mellitus (DM) is a group of a metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion and/or insulin action [1]. Chronic hyperglycemia in diabetes is related to long-term damage and dysfunctioning of several organs of the body [2]. WHO [3] has reported approximately 346 × 10^6 people in the world to suffer from DM. The International Diabetes Federation [4] estimated that in 2030, people with diabetes would rise to 438 × 10^6.

Until now, marine resources in Indonesia have not been widely used as a source of active ingredients for the food and pharmaceutical industries. One source of marine organisms, that was found in Indonesia but has not been used either as a source of food and a source of bioactive ingredients, is seaweed. Gunung Kidul Beach was a potential area for producing both bioactive compounds from seaweed and sponges [5, 6].

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Bioactive compounds from marine brown algae are potential as an antitumor, antifungal, antiviral, antioxidant, antihypertensive and antidiabetic [7, 8]. The polyphenol content of marine algae has pharmacological effects as antioxidants, antibiotics, anti-inflammatory, hypo-allergenic, antibacterial, and antidiabetic [9]. Nwosu et al. [10] stated that phenol extract of seaweed *Palmaria palmate* and *Ascophyllum nodosum* have potential as an antidiabetic agent by inhibiting α-amylase and α-glucosidase activity. Marine algae also have a high content of antioxidants and can be used to stave off free radicals that arise due to the condition of hyperglycemia in diabetic people [11]. The objective of this research was to analyze the activity of *Sargassum hystrix* J. Agardh, 1847 and *Eucheuma denticulatum* N. L. Burman F. S. Collins & Hervey, 1917 extracts obtained from Gunung Kidul Beach of Yogyakarta in inhibiting the activity of α-amylase and α-glucosidase.

2. MATERIALS AND METHODS

2.1 Materials

The main materials used in this study were seaweed, *S. hystrix* and *E. denticulatum* obtained from the coastal of Gunung Kidul, Yogyakarta Indonesia. The sample of seaweed was identified by expert of Plant Taxonomy Laboratory, Faculty of Biology, Universitas Gadjah Mada. Methanol, sodium carbonate, Folin-Ciocalteu's reagent, essential oils and acid gallate were obtained from E. Merck. The *α*-amylase from *Bacillus* sp. type II-A, *α*-glucosidase from *Saccharomyces cerevisiae* type I, p-nitrofenil-*α*-d-glucophiranoside and 3,5-dinitrosalisilat (DNS) acid were purchased from Sigma-Aldrich.

2.2 Marine Algae Extractions

In this study, the extraction of seaweed has been made to obtain an extract containing polyphenols and phlorotannin. The extraction of the seaweed *S. hystrix* and *E. denticulatum* to get polyphenols uses a modification of the method Zhang et al. [12].

Phlorotannin extract was obtained by following a modified method of Chowdhury et al. [13]. Polyphenol powder of 5 g was added to Erlenmeyer flask wrapped with aluminum foil. Afterward, 40 mL of methanol was added and stirred for 2 h, and then allowed to stand for 24 h, and then 20 mL of chloroform was added while stirring for 20 min. The mixture was centrifuged at 3500 revolutions per minute (rpm) for 20 min and the supernatant was separated (1 rpm = 1/60 Hz). A volume of 15 mL aquabidest was added to the supernatant with constant stirring for 10 mins and lipid and non-lipid layers were formed. Non-lipid layer that floats over the lipid layer was separated with the addition of 25 mL ethyl acetate and stirred for 30 min. Subsequently, the mixture was evaporated, freeze-dried and stored at -20 °C before use for further analysis.

2.3 Measurement of Total Polyphenol Content

The total phenols content in seaweed was analyzed using a modified method of Zhang et al. [12]. Gallic acid was used as a standard with concentrations ranging from 0 $mg.mL^{-1}$ to 400 $mg.mL^{-1}$. Serial dilutions of polyphenol extract with concentrations of 3.125 $mg.mL^{-1}$ to 200 $mg.mL^{-1}$ were made. An Aliquot of 200 mL from each solution was added to the test tube, followed by the addition of 1000 mL of Folin-Ciocalteu reagent and incubated for 5 min. After the incubation, 800 mL of 20 % Na_2CO_3 solution was added to the mixture and incubated in dark at 27 °C temperature for 75 min. The absorbance of the incubated mixture was observed at 750 nm wavelength.

2.4 Measurement of Total Phlorotannin Content

The total content of phlorotannin of seaweed was analyzed using a modified method of Koivikko et al. [14]. Phlorotannin extract of 0.1 g was macerated with 200 mL of 85 % ethanol (1:2) in the dark for 8 hours. Phloroglucinol standard solution were made at various concentrations, i.e. 6.25; 12.5; 25; 50; 100 μg . mL^{-1}. Phlorotannin extract was made in a concentration of 1 mg · mL^{-1} following the serial dilution series of concentration, 125 $mg.mL^{-1}$ to 200 $mg.mL^{-1}$. Then, the solution of each sample of 500 mL was pipetted and put into a test tube. Then, 500 mL of Folin-Ciocalteu reagent and 1 mL 20 % Na_2CO_3 were added, and then the mixture was left for 3 min. Furthermore, the solution in a test tube was incubated in the dark room temperature of 27 °C for 45 min, then centrifuged for 10 min at a speed of 3500 rpm. The

absorbance of supernatant was recorded at 730 nm wavelength. A total Phlorotannin content was expressed as phloroglucinol equivalents (PGE) in mg. mg^{-1} extract.

2.5 Inhibition of α-Amylase Activity

Inhibitory activity of *α*-amylase was decided by calculating changes in 3.5-dinitrosalicylate acid into nitro-aminosalicylate utilizing spectrophotometry [15]. A volume of test solution was made from 25 mL of sample extract at differing concentrations and 25 mL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing 13 U mL^{-1} of *α*-amylase. The test solution was mixed using vortex mixer and incubated at 37 °C for 10 min. After that, 25 mL of soluble starch 1 % in 0.02 M sodium phosphate buffer was added into the test solution and it was incubated at 37 °C for 10 min. Then, it was further handled with the addition of 50 mL 96 mM 3, 5-dinitrosalisilat acid (DNS) and incubated for 5 min in water bath. The solution was cooled at room temperature and the absorbance of the solution was recorded at a wavelength of 550 nm. Absorbance values of sample were obtained and used to calculate the percentage (%) inhibition of the enzyme.

$$\%\,inhibition = \frac{K - (S1 - S0)}{K} x100\ \% \quad (1)$$

where:
K= Absorbance of control-blank
S_1= Absorbance of sample with enzyme
S_0= Absorbance of sample without enzyme

2.6 Inhibition of α-Glucosidase Activity

Inhibitory activity of *α*-glucosidase was performed as stated by the method of Mayur et al. [16]. Test solution consists of 50 mL 0.1 M phosphate buffer (KH_2PO_4) pH 7; about 25 mL substrate 0.5 mM p-nitrophenyl-α-D-glucopyranoside (PNP-G), 10 mL of sample at various concentrations and 25 mL of 0.2 U · mL^{-1} *α*-glucosidase. The test solution was combined and incubated at 37 °C for 30 min. The reaction was finished by the addition of 100 mL of 0.2 M Na_2CO_3. Inhibition of enzyme activity was analyzed by the amount of p-nitrophenyl formed by measurement of the absorbance utilizing a microplate reader at a wavelength of 405 nm. Absorbance values of sample were obtained then used to calculate the percentage (%) inhibition of the enzyme as shown in Equation 1.

2.7 Statistical Analysis

The data of the extract concentration versus percent inhibition of the enzyme were plotted to obtain the regression equation. The IC_{50} activity value of *S. hystrix* and *E. denticulatum* extracts against *α*-amylase and *α*-glucosidase were achieved from the regression equation. The IC_{50} values were analyzed statistically with one-way analysis of variance using the SPSS (Statistical Package for Social Sciences, IBM, USA) at 95 %.

3. RESULTS AND DISCUSSION

3.1 Total Polyphenol and Phlorotannin Contents

The data of polyphenols and phlorotannins extracts along with the content of each compound was shown in Table 1. The total phenol content of *S. hystrix* and *E. denticulatum* was 3.17 g GAE per 100 g extract and 0.33 g GAE per 100 g extract, respectively. These results were consistent with the previous reports of Kumar et al. [17]. The research of Cox et al. [18] showed that the brown algae (*Himanthalia elongata*) has a higher total phenol, which was (151.33±6.75) mg $GAE.g^{-1}$ extract. Damongilala et al. [19] showed that *P. pavonica* extracted using 60 % methanol resulted in the total phenols content of 4.98 mg $PGE.g^{-1}$ extract to 5.87 mg $GAE.g^{-1}$ extract. The differences in total phenol content could be influenced by intrinsic factors, such as age, type, etc., and extrinsic factors namely tidal cycles, salinity, etc. [20].

Table 1 showed that total phlorotannin content of *S. hystrix* and *E. denticulatum* calculated as 0.02 g PGE per 100 g extract and 0.02 g PGE per 100 g extract, respectively. According to Koivikko et al. [21], only brown algae contained phlorotannin. However, based on this study, phlorotannin content of the red alga *E. denticulatum* was detected although it was not higher than *S. hystrix*. Phlorotannin content is specific to each species [22], factors. i.e. age, species, and environment (location, season, waves, presence or absence of light, salinity, UV radiation, the presence or absence of herbivores and nutrients) could be the reason of the Phlorotannin content. Likewise, Gunung Kidul coastal area is a

Table 1. Yield and total content of polyphenols and phlorotannin extracts from *S. hystrix* and *E. denticulatum*

Sample		Extracts	
		Polyphenols	**Phlorotannin**
Sargassum hystrix	Yield	15.47 %	2.36 %
	Total content	3.17 g GAE per 100g	0.021 g PGE per 100g
Eucheuma denticulatum	Yield	12.40 %	8.24 %
	Total content	0.33 g GAE per 100g	0.018 g PGE per 100g

rocky shore with longer emersion cycle; therefore *Sargassum* species at Gunung Kidul has longer exposed to solar radiation and generally has higher phenolic content. Moreover, Gunung Kidul beach has a high waves so that the seaweed that grows there tends to have high phlorotannin content [23].

3.2 Inhibition of α-Amylase Activity

Based on Fig. 1. the highest inhibitory activity was observed in polyphenols of *S. hystrix* (94.09 %). On the other hand acarbose, polyphenols and phloroglucinol of *E. denticulatum* showed a higher inhibitory activity (88.81 %, 85.84 %, and 85.92 %, respectively) compared to phlorotannin of *S. hystrix* (68.39 %) and phlorotannin of *E. denticulatum* (69.02 %). Polyphenols of *S. hystrix* at these concentrations showed higher inhibitory activity compared to polyphenols of *E. denticulate*, phlorotannin of *S. hystrix* and phlorotannin of *E. denticulatum*. According to Kunyanga et al. [24], phenolic compounds were able to bind to the active site of *a*-amylase. Bioactive components group of phenolics such as anthocyanins, flavonols, flavones, flavanones, gallic acid, vanillic acid, quercetin and trans-cinnamic have been reported to have inhibitory activity against the activity of *α*-amylase [25, 26]. The ability of both seaweeds in inhibiting *α*-amylase was supported by Firdaus et al. [11]. In addition, Lamela et al. [27] reported hypoglycemic activity of *Eucheuma* sp.

Table 2 exhibited the IC_{50} of inhibition activity of *α*-amylase by *S. hystrix* and *E. denticulatum* extracts, acarbose, and phloroglucinol. There were no significant differences between IC_{50} values of acarbose, phloroglucinol and polyphenol of *S. hystrix*. IC_{50} values displayed higher activity compared to other marine algae, for example chloroform extract of *Chaetomorpha aerea* (IC_{50} = 408.9 μg.mL^{-1}) and methanol extract of *Chlorodesmis* (IC_{50} = 147.6 μg.mL^{-1}) [28]. However, it was relatively lower than *Ascophyllum nodosum*. Senthil Kumar & Sudha [29] informed that IC_{50} value of water extract of marine algae (*S. policystum, R. corticata* and *G. lactuca*) for *α*-amylase inhibtion was 60 μg.mL^{-1}, 67 μg.mL^{-1}, and 82 μg.mL^{-1}, respectively. Nwosu et al. [10] reported that methanol extract of *A. nodosum* has a smaller IC_{50} value (0.1 μg.mL^{-1}).

3.3 Inhibition of α-Glucosidase Activity

Inhibition activity of *α*-glucosidase is shown in Fig. 2. These results displayed similar results for acarbose and polyphenols of *S. hystrix* in inhibiting *α*-glucosidase activity, followed by phloroglucinol, polyphenols of *E. denticulatum*, phlorotannin of *E. denticulatum*, and phlorotannin of *S. hystrix*. The ability of polyphenols to inhibit α-glucosidase in the digestive tract and activation of glucose uptake lowered blood glucose [12, 15]. Anthocyanin, especially polyphenols, flavonols, proanthocyanins, and phenolic acids, significantly suppress the elevated blood glucose and reduce the rate of digestion of sucrose and glucose absorption in the intestine [30]. The workings of a polyphenol are similar to acarbose, which extends the time revamp of carbohydrates and inhibit the absorption of glucose [31].

Alpha-amylase and alpha-glucosidase are enzymes that are closely associated with diabetes mellitus. Phenols are one of the bioactive components that can inhibit the action of *α*-amylase and *α*-glucosidase [32, 33]. Polyphenols can inhibit the enzyme in the breakdown of carbohydrates into glucose. The content of phenol has an inhibitory effect on *α*-amylase through bond hydroxylation and ring substitution on *β*. The principle is similar to acarbose inhibition, i.e., by generating delays and disaccharide carbohydrate hydrolysis and absorption of glucose and inhibiting the metabolism of sucrose into glucose and fructose [34]. Besides, that phlorotannin is one of the phenolic components which can also inhibit the

work of the α-amylase and α-glucosidase [35, 36]. As polyphenols, phlorotannin inhibits enzymes work in the breakdown of carbohydrates into glucose. The principle was also similar to acarbose inhibition; that was, to produce a delay, hydrolysis and absorption of carbohydrates and disaccharides inhibit the metabolism of glucose and sucrose into glucose and fructose [34].

4. CONCLUSION

Seaweed *S. hystrix* has more potential as an antidiabetic substance compared to *E. denticulatum*. The results displayed that polyphenols *S. hystrix* [IC_{50} = (0.58 ± 0.01) mg.mL^{-1}] can inhibit α-amylase similar to like acarbose [IC_{50} = (0.53 ± 0.00) mg.mL^{-1}] and phloroglucinol [IC_{50} = (0.56 ± 0.01)

Table 2. Inhibitory activity (IC_{50}) of polyphenol and phlorotannin extracts from *S. hystrix, E. denticulatum*, acarbose, and phloroglucinol against α-amylase and α-glucosidase.

Inhibitors	$IC_{50}$of α-amylase (mg · mL^{-1})	$IC_{50}$of α-glucosidase (mg · mL^{-1})
Acarbose	0.53 ± 0.00[a]	0.61 ± 0.01[a]
Phloroglucinol	0.56 ± 0.01[a]	0.56 ± 0.05[a]
Polyphenols *S. hystrix*	0.58 ± 0.01[a]	0.59 ± 0.02[a]
Polyphenols *E. denticulatum*	1.43 ± 0.19[b]	1.43 ± 0.19[d]
Phlorotannin *S. hystrix*	3.29 ± 0.12[d]	0.78 ± 0.04[b]
Phlorotannin *E. denticulatum*	1.92 ± 0.14[c]	0.86 ± 0.06[c]

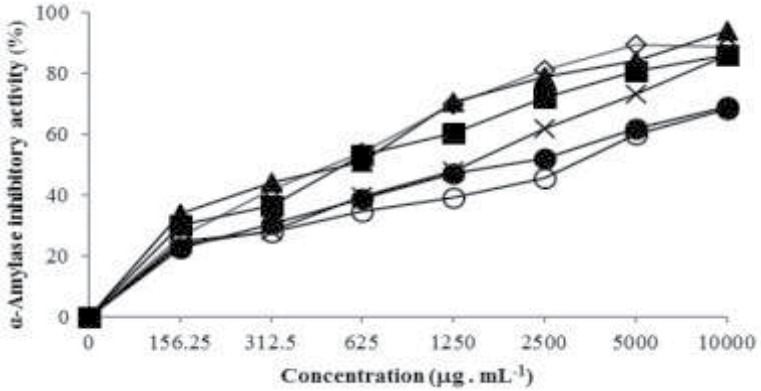

Fig. 1 Effect of sample concentration (▲: polyphenol *S. hystrix*, ´: polyphenol *E. denticulatum*, ○: phlorotannin *S. hystrix*, ●: phlorotannin *E. denticulatum*) and control (◊: acarbose, ■: phloroglucinol) on inhibition activity of α-amylase.

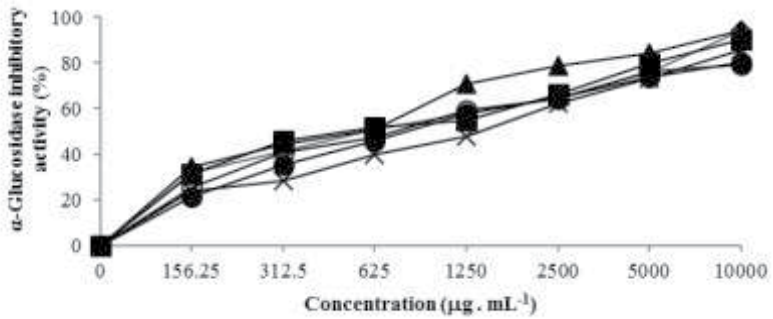

Fig. 2 Effect sample concentration (▲: polyphenol *S. hystrix*, ´: polyphenol *E. denticulatum*, ○: Phlorotannin *S. hystrix*, ●: Phlorotannin *E. denticulatum*) and control (◊: acarbose, ■: phloroglucinol) on inhibition activity of α-glucosidase.

mg. mL^{-1}]. Inhibitory activity of polyphenols *S. hystrix* [IC_{50} = (0.59 ± 0.02) mg.mL^{-1}] in inhibiting α-glucosidase is also similar to acarbose [IC_{50} = (0.61 ± 0.01) mg.mL^{-1}] and phloroglucinol [IC_{50} = (0.56 ± 0.05) mg.mL^{-1}].

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Research Article

Impact of Global Warming on South Asia Low Pressure and Regional Cloud Cover

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Abstract: Clouds perform a very unique function in whole of the atmospherics. They respond differently to the same scenario. Analyzing more than second half of the last century synoptic climatic data, it is observed that climatic parameters are sensitive to the increasing temperature. In North Atlantic cloud cover increases with temperature while it works the other way in South Asia. By using the Center of Action (COA) technique, it is suggested that the intensity of South Asia Low pressure (spread over from north India to Saudi Arabia) has been increasing from mid 70s. It is also found that the declining trend in regional cloud cover has some links with weakening low pressure system.

Keywords: Cloud cover, Center of Action, South Asia Low pressure, Indian Ocean

1. INTRODUCTION

Clouds are condensed product of water vapors, frequently observed phenomenon. They are estimated to cover between 60 and 70 % of the globe at any given time. They are directly linked to a large variety of weather phenomena and play's an important role in climate, affecting both radiation fluxes and latent heat fluxes, but the various cloud types affect climate in different ways. Marine stratus and stratocumulus clouds (MSC) have an albedo of 30-40%, while maintaining a cloud-top temperature not much below the sea-surface temperature (SST). MSC therefore have a cooling effect on climate (negative cloud radioactive effect, CRE). Randall [1] estimated that a 4% increase in MSC cover could compensate for a 2-3°C global temperature rise. By contrast, high (cirriform) clouds are thinner and colder, so their long wave effect dominates, giving them a positive CRE.

The changes in cloud cover linked with climate change and how such feedback change with climate is one of the most challenging aspects of predicting future climate and anthropogenic effects it may accompany. There have been several studies of cloud cover change over land in last decade. People have employed historical data during hot and cold periods that lasts from decade to more than a century [1, 2, 3, and 4]. Hameed [2] suggested that secular changes in the strengths of three permanent high/low pressure systems, the North Pacific high, the Icelandic low, and the Azores high, are in part related to secular changes in global climates, that is, changes in global mean surface temperature. He further suggested that the climate –induced change in cloud cover for certain regions is related to the strengths of adjacent high/low pressure systems. Hahn [5] observed 22 decadal-scale variations in cloud cover for most cloud types. He also noticed (while looking at smaller regions) possible increase in total cloud cover in central Pacific, while possible decline is seen stratiform cloud cover of persistent stratocumulus clouds. The decline in stratocumulus clouds is accompanied by an increase in SST between 1954 and 2008. Lower tropospheric stability and sea level pressure show long-term increase.

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Norris [5] also suggested a decreasing decadal trend of 1.9% of sky cover in Staturs cumulus (Sc) cloud cover from 1952-97, which can be due to a higher trade inversion, warmer SST, and/or weaker trade winds. During this period the Inferred net outgoing radiation has decreased by 0.8 Wm^{-2} in Sc regions (0.045 Wm^{-2}globally). Jaswal [1] observed a decrease in total cloud cover over large parts of India during 1961-2007. While trends in rainy days are similar to cloud cover across periods, trends in diurnal temperature have regional to seasonal preferences over the country. In Warren's [6] previous work, analyzing cloud cover over land they set forth criteria for the examination of surface observations for trends and inter-annual variations of cloud cover. The work concluded that total cloud cover was declining slightly over the global land areas and hinted that cumuliform clouds may be increasing at the expense of stratiform clouds at low and middle levels. High cloud amount was also shown to be decreasing. Dim [7] compared two satellite datasets [Advanced Very High Resolution Radiometer (AVHRR) and International Satellite Cloud Climatology Project (ISCCP)], and found a decrease in cloud cover over land, although the character of the change was different in the satellite data—[8] the observed decline was mainly caused by a decrease in low cloud cover.

These and other studies suggest the continuous updating is required in cloud data and models. Cloud cover has strong temporal and spatial behavior globally. In January cloud may have positive correlation with temperature but the same may reverse in July. In tropics and subtropics cloud cover goes against the increase in temperature while in extra tropics it seems to go with the temperature [9]. The factors which influence cloud feedback to the atmosphere may have been temperature, lower tropospheric stability, relative humidity, sea surface pressure. The uneven solar heating from equator to poles throughout the annual cycle of the Earth has the leading role. The above mentioned studies do give us some insight of cloud dynamics and their variation over the year. These observed changes in cloud amount have some localized atmospheric anomalies which fluctuates the balance in atmospheric layers at different pressure levels. Some permanent/semi-permanent high/low pressure systems might also be responsible for changing cloud amount.

South East Asia has a very different ocean--atmosphere interaction than other well-known western oceans and their climates. The sole sink and source of solar/terrestrial incoming /outgoing radiation is Indian Ocean. Other than South the Indian Ocean is surrounded by geographically different land masses. In North there is an 1800 km long and over 5000 meters high mountain range (Tibetan plateau). In East, there is a cluster of a few islands and Australian continent working as a sparse boundary between Pacific and Indian Ocean. The Western side consists of East African plains. Due to this structural setting climate of south Asia is dominated by temperature gradient. Sometime these gradients are due to persistent cloud cover on the same surface as well as different surfaces (ocean-land). In the past, warm periods have shown an increase in cloud cover in contrast to cold period [10]. As described above the climate of south Asia has its differences and there is no previous attempt to understand the change in cloud cover to relate with other aspects of atmospheric circulation, that is, the change in global climate has altered the cloud cover. This would seem to be a particularly relevant endeavor, given the importance of understanding how clouds interact with the change in climate. For this purpose we have averaged the data over central and south India and have found a decrease in cloud cover with the increasing temperature and this decrease in cloud cover is also observed in increasing low heat, suggesting a possible link between regional cloud cover change and the strengths of related low pressure systems.

2. Radiative cloud effects

Clouds interact with radiation from both sides. If clouds form on an otherwise sunny day the maximum temperature near the surface will be lower due to the reflection of sunlight by clouds (from side away from earth), in comparison to the temperature without clouds. Cloud cover (from side towards earth) traps the outgoing terrestrial radiation which leads to warming the covered area. Likewise, if the sky is covered by low clouds at night the near-surface temperature will not drop as low as under clear sky conditions due to the trapping of terrestrial radiation by the clouds.

The overall impact of clouds on the radiation budget can be measured by comparing the radiative fluxes in cloudy to those in clear-sky conditions.

The difference between the two has been termed cloud radiative forcing [11] and can be defined separately for the solar (short wave radiation) and terrestrial (long wave radiation) parts of the spectrum. The major source for radiative fluxes is satellite data, which measure radiation at the top of the atmosphere (TOA). There have been numerous studies 12, 13, 14, 15, 16] to establish the effect clouds have on the TOA radiation budget many of which are based on satellite data from the Earth Radiation Budget Experiment [17].

3. Datasets

Investigating cloud-cover change in south Asia associated with local climate change, we have adopted global mean temperature and cloud-cover data for the period of 1948 to 2012 from an online facility provided by National Center for Environmental Prediction/National Center for Atmospheric Research (NCEP/NCAR). For current investigation monthly means cloud cover and global temperatures with a $0.52^{o} \times 0.52^{o}$ latitude-longitude resolution data is used. Land area cloud cover is included (falling under the region from $8^{o} - 22^{o}$N to $73^{o} - 83^{o}$E).

Additional dataset consists of the intensities of the subtropic pressure system situated in central and southern India and Northern Indian Ocean is used. These were evaluated by Iqbal [11] from sea level pressure (SLP) data. The pressure index *Ip* of a low pressure system is defined as an area-weighted pressure departure from a threshold value over the domain (I, J):

$$I_{p,\Delta t} = \frac{\sum_{i=1}^{I}\sum_{j=1}^{J}\left(P_{ij,\Delta t} - P_t\right)\cos\phi_{ij}(-1)^M \delta_{ij,\Delta t}}{\sum_{i=1}^{I}\sum_{j=1}^{J}\cos\phi_{ij}\delta_{ij,\Delta t}}$$

Similarly, the latitudinal index, is defined as:

$$I_{\phi,\Delta t} = \frac{\sum_{i=1}^{I}\sum_{j=1}^{J}\left(P_{ij,\Delta t} - P_t\right)\phi_{ij}\cos\phi_{ij}(-1)^M \delta_{ij,\Delta t}}{\sum_{i=1}^{I}\sum_{j=1}^{J}\left(P_{ij,\Delta t} - P_t\right)\cos\phi_{ij}(-1)^M \delta_{ij,\Delta t}}$$

Where $I_{\phi,\Delta t}$ is the latitudinal index (SALT) of South Asia Low pressure (SALP), $P_{i\,j,\,\Delta t}$ is the SLP value at grid point (i, j) averaged over a time interval Δt, in this case monthly SLP values are taken from NCEP/NCAR reanalysis, P_t is the threshold SLP value, φ_{ij} is the latitude of the grid point (i, j). Here M = 0 for the High and 1 for the Low is used. $\delta = 1$ if $(P_{i\,j,\,\Delta t} - P_t) > 0$ and $\delta = 0$ if $(P_{i\,j,\,\Delta t} - P_t) < 0$, this makes sure that the pressure difference is due to the Low pressure system. The intensity is thus a measure of the anomaly of the atmospheric mass over the section (I, J) [2]. The domain of the South Asia Low pressure (SALP) was chosen as 10^{o}N to 35^{o}N and 35^{o}E to 95^{o}E, since this is the region where the Inter tropical Conversion Zones (ITCZ) moves to in summer, the rest of the year it is found near the equator (on either side north or south). The Low pressure and their threshold values P_t were chosen by examining their geographical ranges in NCEP/NCAR reanalysis data over the period 1948-2006. Similarly, the South Asia longitudinal index (SALN) is defined. Cloud cover data is examined by performing smoothing to remove the short term fluctuations without distorting the dynamics of the variables. Only daytime values are analyzed, in order to avoid any day/night sampling biases. [The analysis was also done using the day/night averages, and very similar results were obtained].

4. Analysis

Different surface types have different balances between incoming radiation, and outgoing heat

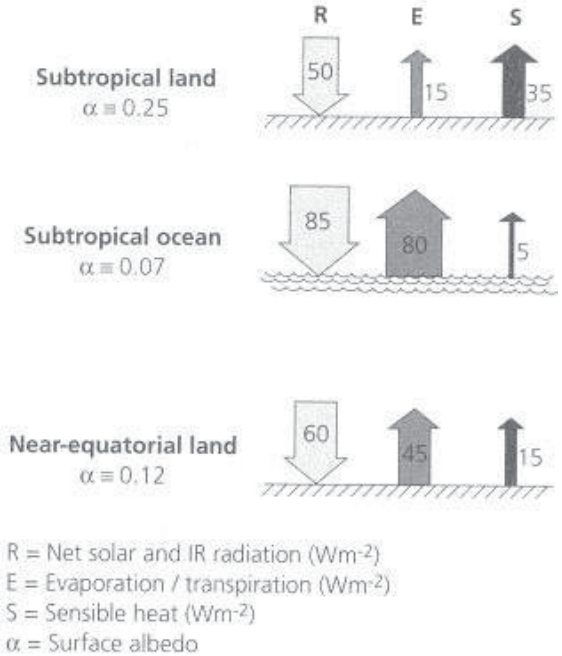

Fig. 1. Effects of three different surfaces on heat balance. After [3]

losses through sensible heat and evapotranspiration displayed in Fig.1. Subtropical land has higher sensible heat than subtropical ocean; the land shows a prompt response to any increase in temperature than ocean. As a result, over land we have low pressure systems developed in South East Asia during summer monsoon. Prevailing pressure system causes monsoon winds to blow from the southeast from April to October. From November to March they blow from the northeast. This dry air prevails to create the dry season. The monsoon brings rains when it blows from the southwest across the warm waters of the Indian Ocean. This wind is blown due to the presence of Low pressure system developed due to the mid troposphere heating of the Tibetan plateau from March to May. The change in the direction and intensity of wind is due to locally developed pressure systems. These winds also carry clouds formed at neighbouring seas and create a cover for precipitation.

Over the years (mostly since mid 1950s) cloud cover and temperature have shown some consistent variability together in Fig. 2 & in Fig. 3. The negative correlation found between the two variables displayed in (Fig. 4a, b, c) may be due to the type of stratocumulus (MSC) cloud found at low latitudes. In north Atlantic the cloud cover goes with the increasing temperature [14] which is contrary to our findings for south Asia. There can be two possible mechanisms for this negative correlation, one is when the landmass heats up (via solar radiation) the increased sensible heat carries less water vapours and results in decreased cloud cover. For the other possibility this has been found in numerous studies [15, 21, 7] that marine stratiform cloud cover should and does correlate negatively with SST. The hypothesized mechanism for this relationship is a destabilization of the lower troposphere by the warming SST, reducing static stability and entering more dry air into the cloud layer. Taking this possibility further, the destabilization doesn't stop there but it also decreases the intensity of the associated low pressure as shown in Fig. 5. In North Atlantic the sea level pressure and temperature have similar association [15]. Although a correlation does not necessarily establish the cause-n-effect relationship, but still it gives a clue of probable association. The implied result from this analysis is that the decrease in the intensity of low pressure may decrease the cloud cover Fig. 6. The longitudinal component of SALP

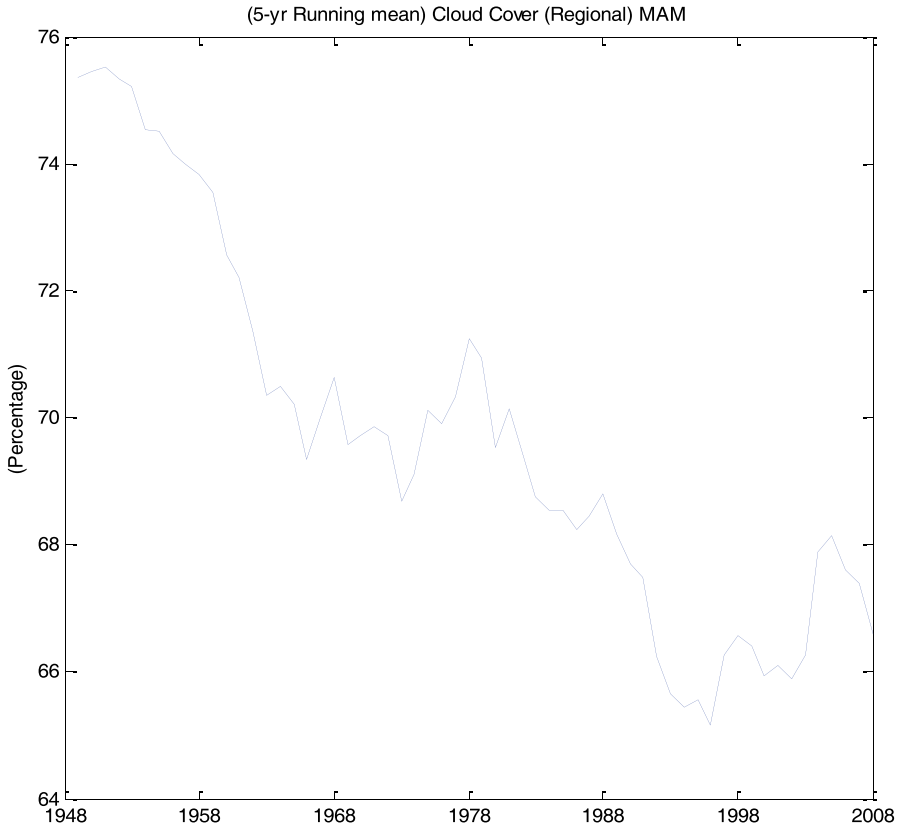

Fig. 2. Decadal variation in cloud cover during pre-monsoon for investigated region

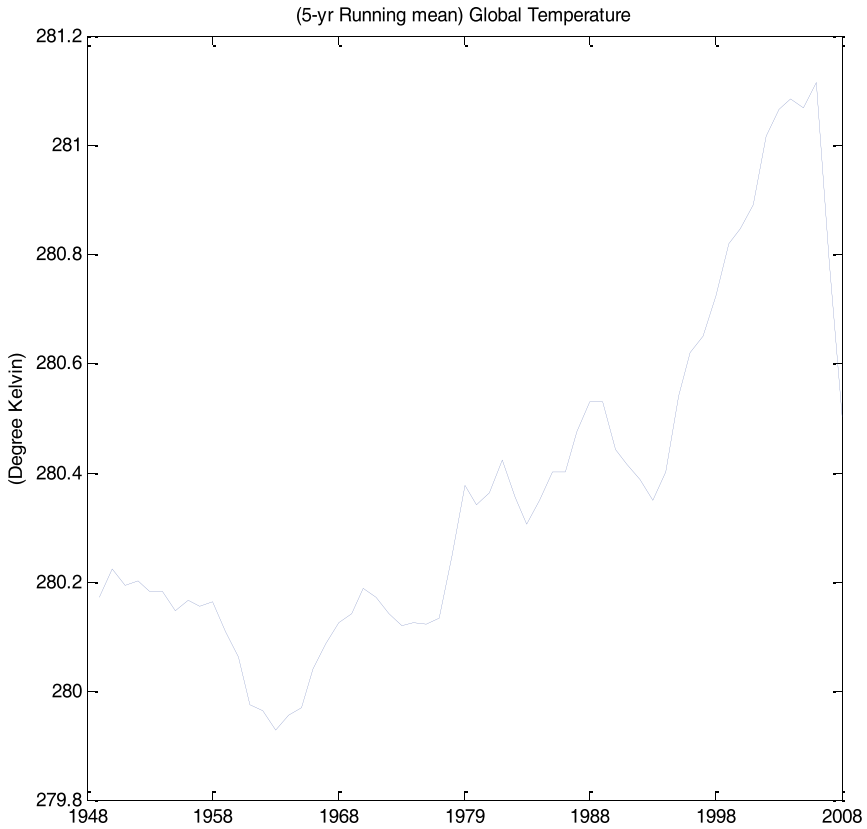

Fig. 3. Global temperature increase for the investigated duration

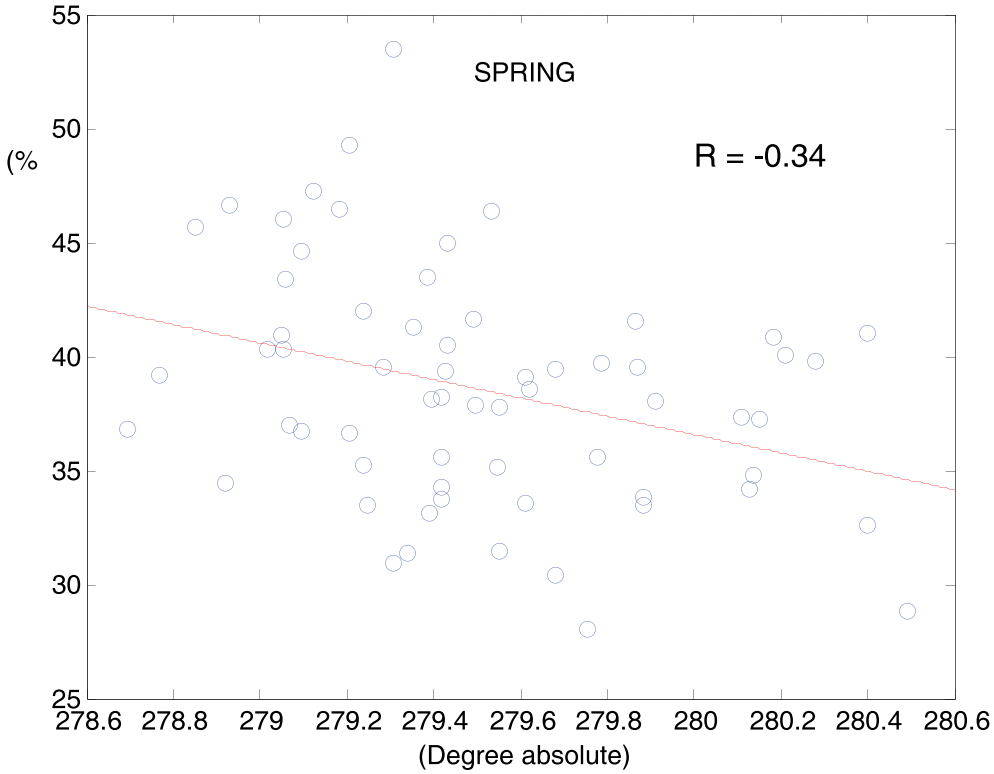

Fig. 4a. Global temperature Vs Regional cloud cover (Spirng)

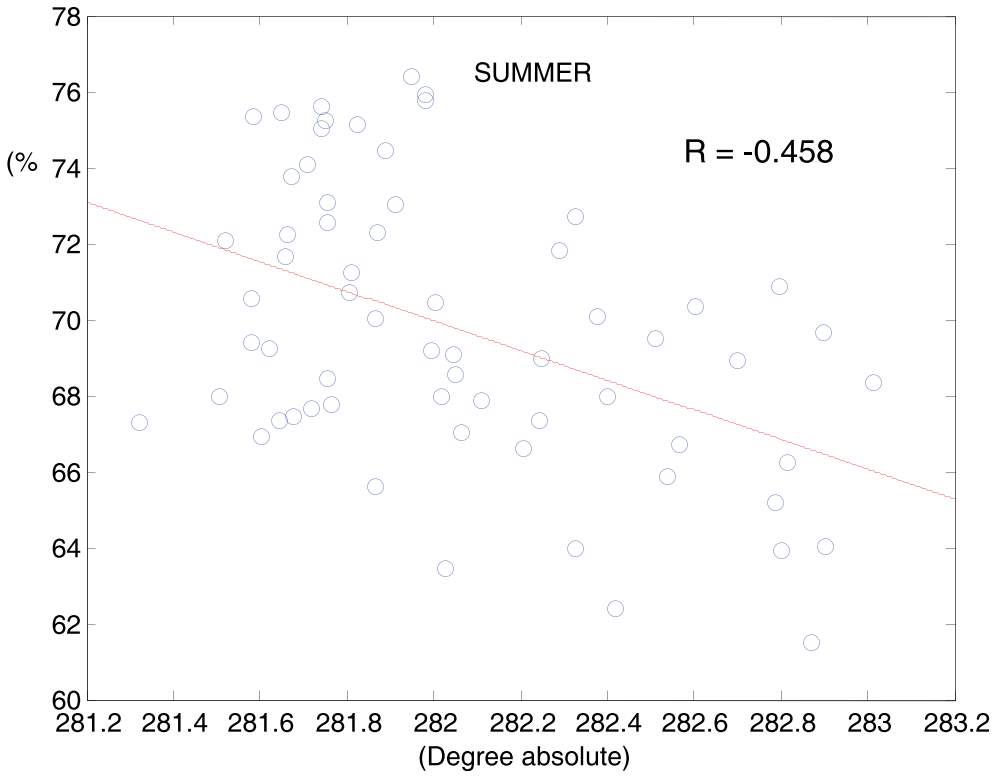

Fig. 4b. Global temperature Vs Regional cloud cover (Summer)

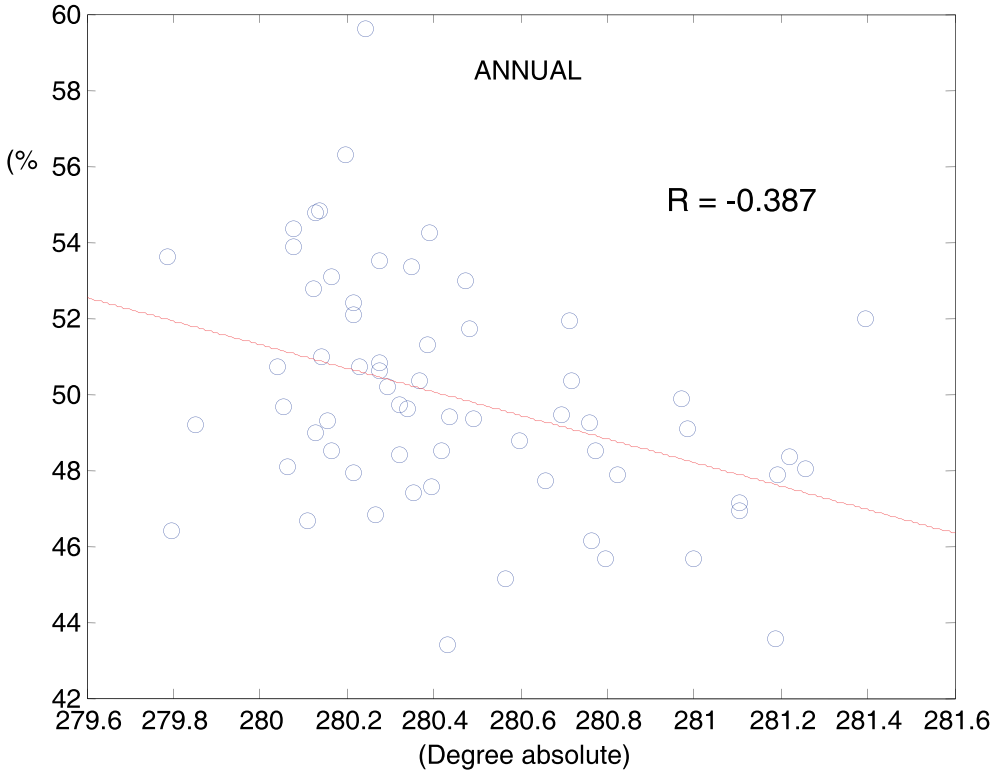

Fig. 4c. Global temperature Vs Regional cloud cover (Annual)

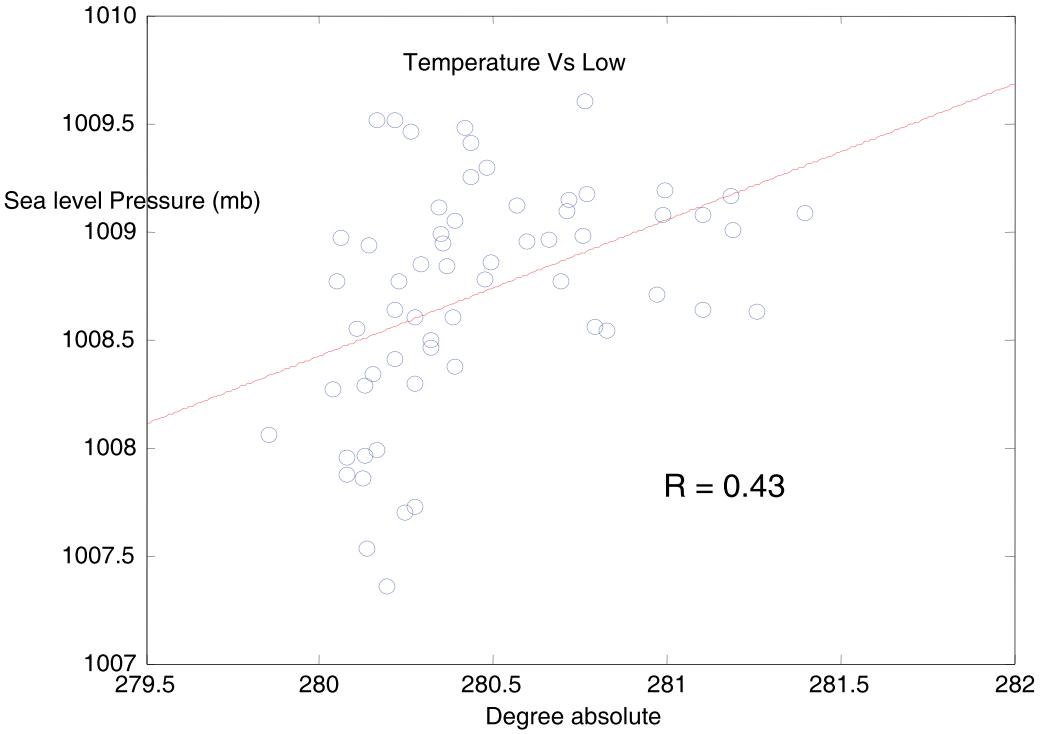

Fig. 5. Temperature increase decreases the intensity of low pressure

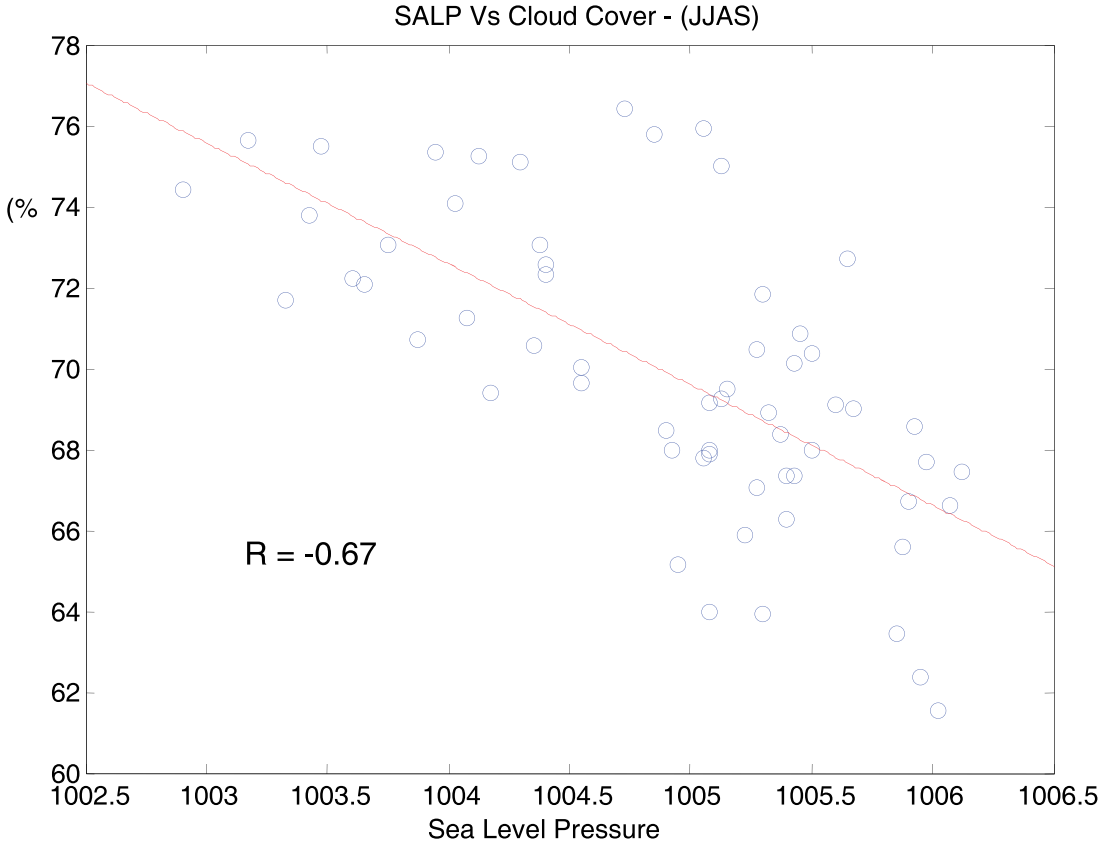

Fig. 6. Cloud cover decreases with decreasing low pressure system Negative correlation (p = 0.05)

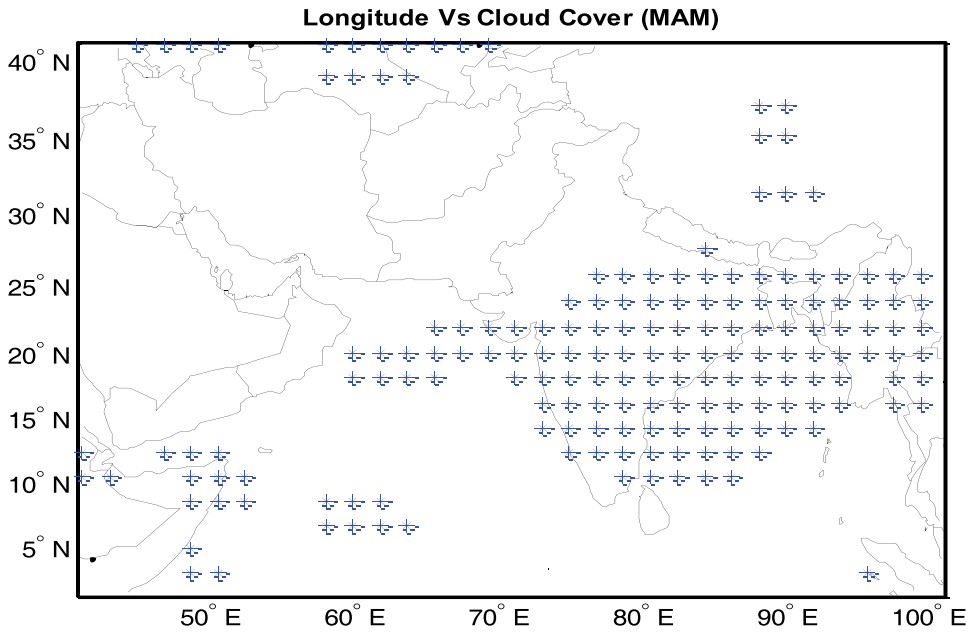

Fig. 7. SALN has significant negative correlation with cloud cover (p = 0.05) in pre-monsoon season

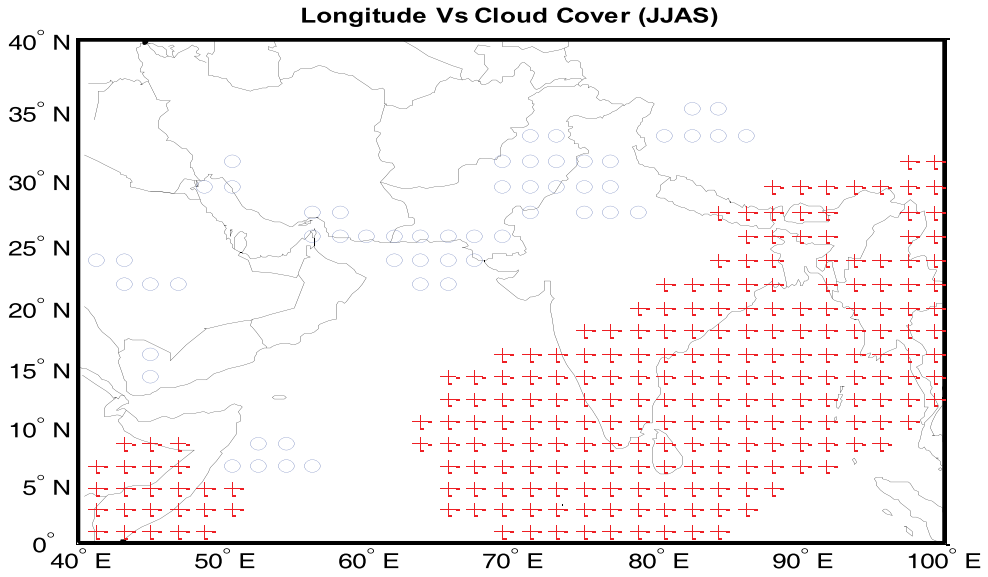

Fig. 8. SALN has significant positive (red) correlation with monsoon cloud cover (p = 0.05)

seems to play the major role in the above mentioned relationship. At pre-monsoon (MAM) stage of the low pressure in Bay of Bengal, eastern coast of India along with southern and central India are all under the influence of South Asia's longitudinal component (SALN), in the form of decreased cloud cover (Fig.7). In the following monsoon season (JJAS) this pressure system develops and supports the cloud formation (Fig.8) for monsoon precipitation.

5. Concluding remarks

Landmass tends to heat and cool quickly in comparison to oceans. This decreases lower trophospheric stability at different pressure levels (at 700, 850 and 1000 mb). As a result the pressure gradient so formed influences the formation of cloud cover at different pressure levels.

The land cloud cover is created over ocean (i.e.,

Indian Ocean) and then travels to its neighbouring low pressure. Cloud cover is more sensitive to increase in temperature than stable or decreasing temperature (Fig.1 & Fig. 2), especially lower level clouds. On the other side decreasing intensity of low pressure seems to decrease cloud cover. As other regional climates, South Asian climate also appears to depend upon the development and intensity of low pressure systems which also contributes to the Indian summer monsoon season.

The inter-annual and decadal scale decrease in cloud cover is attributed to the increase in temperature. As the persistent declining trend of low pressure system does not favour the formation of cloud cover, it also contributes partly, in decrease of monsoon rainfall over South Asian region. Since increase in temperature diminishes both, the associated low pressure system and the regional cloud cover, with every degree rise in regional temperature the climatic parameters will become more severe.

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Research Article

Impact Assessment of School WASH Programme on Students' Health and Hygiene Conditions in Rural Mardan, Pakistan

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Abstract: The implementation of water, sanitation and hygiene (WASH) programme in schools with true its spirit can largely solve the issues related to poor water quality, sanitation, health and hygiene conditions, which resultantly improve children attendance and hygienic environment in schools. In the present study, an attempt has been made to investigate the impacts of WASH programme on student health and hygiene condition in selected schools in district Mardan, Khyber Pakhtunkhwa, Pakistan. Social surveys including questionnaire, personal interview, focus group discussion (FGD) and personal observation were carried out to collect the data related to WASH activities. Drinking water samples were collected and analyzed from the targeted schools for different physical, chemical and bacteriological parameters including electrical conductivity (EC), pH, total dissolved solids (TDS), turbidity, *E. coli,* and *total coliform*. Statistical analyses, like descriptive statistics (i.e. ANOVA) were applied to analyze the data. The data collected using different social survey methods showed that the hygiene conditions in schools were not up to the standard, number of latrines in each school was not enough and no hand washing with soap was found practicing in each school. As a result, the number of children suffering from water borne diseases including diarrhea was higher as compared to other diseases. Water quality descriptive statistical analysis revealed that all the parameters were significantly different ($P < 0.05$) and uniformly contribution to the overall contaminant loads in the water samples, except the water pH values. Findings from the present study suggested that WASH programme is effective for promoting health and hygiene education in school going children.

Keywords: WASH programme; Water quality; Health and Hygiene; Sanitation; Social survey

1. INTRODUCTION

Water Sanitation and Hygiene (WASH) in schools is related with water, sanitation and hand washing facilities in schools along with basic hygiene education. Availability of WASH services in schools has a positive impact on children health and education [1]. Having access to clean and safe drinking water and sanitation facility is one of the basic human rights which must be fulfilled at any cost, because lack of access to basic WASH facilities is known to be one of the major causes of child mortality [2]. It has been reported that 2.5 billion people living globally do not have access to proper sanitation facilities as a result of which one billion people are forced to practice open defecation [3], which contaminate the environment badly and causes about 0.577 million deaths annually [4]. Recently, it has been estimated that infections which children contract in schools will lead to infections in up to half of their household members and that 88% of diarrheal diseases are caused by unsafe water supply, inadequate sanitation and inappropriate hygiene [1].

The presence of school sanitation service and proper hygiene facilities is very important for child attendance, particularly for girls' students during natural cycle days [5, 6]. The use of improved sanitation facilities can reduce the rate of diarrhea cases by 34%, hand washing with soap after toilet use can reduce the incidence of diarrhea by nearly

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40% [7]. Also, Rabie and Curtis [8] compared the results from different countries and found that hand washing with soap can eliminate the risk of respiratory infections by 16%. The ratio of absenteeism caused by gastrointestinal related diseases in developed nations revealed that the number of days lost can drop up to 50% due to improved hygienic environment in schools [9]. Globally, 25% students of the school dropout were reported even before the completion of primary schooling and in sub-Saharan Africa region most of the girls are not even enrolled [10]. Thus, the access to safe drinking water, proper sanitation facility is basic human right, which must be fulfilled for every human being [7, 11].

In Pakistan, nearly 15 million people drinks unsafe water and over 93 million people do not proper sanitation facilities, which resulted of promoting open defecation and the transmission of pathogens [12]. Due to lack of proper WASH facilities in Pakistan the mortality rate cause to death of 110 children every day particularly school going children are more vulnerable to WASH related diseases [13]. Therefore, it is necessary to ensure that schools children have proper improve access to WASH facilities for better children growth, reduce school dropout ratio and subsequently better performance [14]. Unfortunately, many schools in rural areas still lack proper WASH facilities affecting the health of children [13].

Integrated Rural Support Program (IRSP), a local Non-Governmental Organization (NGO) in district Mardan (Pakistan) has gained distinctive position and is a national level developmental agency for successful completion of Community Led Total Sanitation (CLTS) projects in District Mardan in the year 2004. Afterward, IRSP has taken a great step to work for WASH at different schools in rural Mardan and after detailed surveys of the area and interviews with school head and teachers, a school WASH programme was introduced in selected schools mainly in girls and carried out different activities for the better health promotion and hygiene. The impacts of these interventions on rural children's health and hygiene conditions in district Mardan have not been reported in literature so far. So, the purpose of this study was to evaluate the impact of school WASH programme on the overall health and hygiene conditions of the students. Also, to evaluate the role of school WASH activities on students' attendance and dropout ratio and the status of schools water quality available in schools.

2. MATERIALS AND METHODS

2.1. Study Area

Tehsil Takhtbhai in district Mardan is located in Khyber Pakhtunkhwa Province of Pakistan having latitude and longitude of 34°16'54.83" and 71°55'41.88", respectively. It is 15 kilometers away from Mardan city and is famous for wheat, maize and sugarcane growth. After detailed survey and physical visits the following Union Councils (UCs) of Takhtbhai were selected for this study: Madhay Baba, Naray, Saroshah, and Takkar. Figure 1 presents the detailed location of the targeted UCs.

2.2. Secondary Data Collection

Data related to number of schools in each union council in which WASH interventions were carried out by IRSP, which were collected through surveying and from District Education Office Mardan. After having complete school profile, need assessment of schools and different practices e.g. hand washing and latrine usage was considered for comparative analysis.

2.3. Questionnaire Survey

Detailed questionnaire about WASH facilities at schools, and to assess the student's knowledge level about health and hygiene was assessed using pre-designed questionnaire. The questionnaire contained both close and open ended questions to gather information about the role of school WASH club, pamphlets, brochures, school trainings, hygiene walks and other associated activities to improve the health and hygiene conditions of the students especially girls.

2.4. Focus Group Discussion

In every school, focus group discussion (FGD) was organized for the students to know the impacts of health and hygiene related activities on the hygiene conditions of students. FGD related to the WASH facilities at different schools were discussed in detail with the students. The students participated and responded in a very positive way by sharing

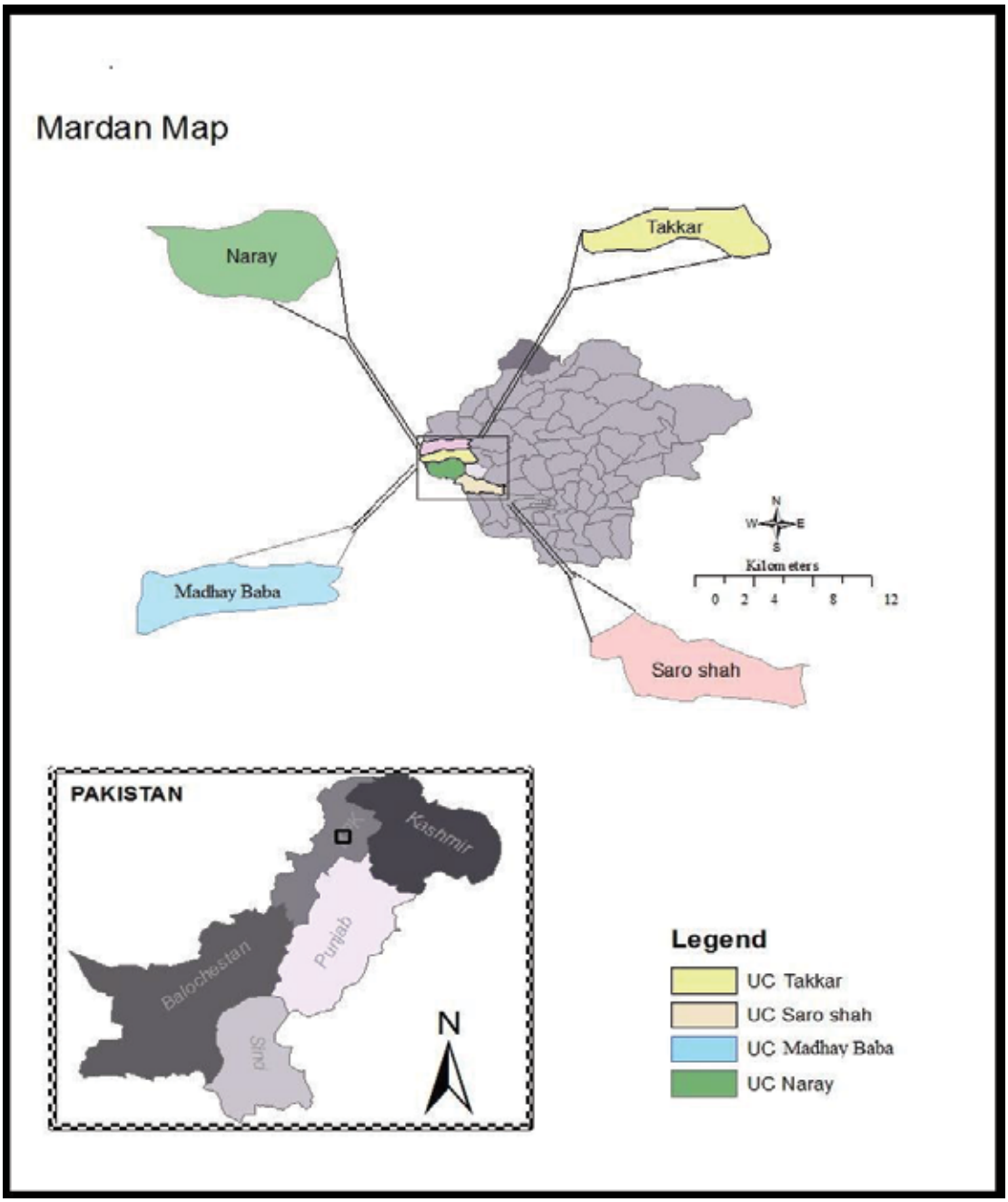

Fig. 1. Location map of district Mardan and also highlighted the targeted UCs

their problems related to hygiene conditions persisting in their schools.

2.5. Personnel Interviews

Interviews with the teachers and students about their opinions on the impact of the above mentioned health and hygiene activities on student's health and attendance was carried out inside the schools. Similarly, interviews with the NGO workers were held about their criteria of selecting school for WASH programme and what they are expecting from this project and what they achieved so far.

2.6. Water Quality Analysis

For children's safety and for better health promotion the drinking water used by children at targeted schools were sampled using sampling bottles and tested for contamination at IRSP lab in Mardan. Water quality parameters including E. coli, total coliforms, pH, turbidity and conductivity

were analyzed through pH meter (HI 98129), electric conductivity meter (HI 98130 combo Hanna instrument) and turbidity meter (2100 P ISO HACH) by taking 100 mL water in cuvette and placing it inside the meters, while E.coli and total coliforms were detected by using filter machine (SIMCO model # 800). For this purpose, water sample was analyzed for E. Coli and total coliform detection needs to be filtered using filter machine. After filtration the Milli pore filter paper is placed in the EC plates which needs 2 mL distilled water for activation and are shifted to the incubator, where the temperature and timing of the incubator is set at 37 °C for about 24 hours. After incubation the colonies formed were counted to know total number of E. coli and total coliforms formation.

2.7. Statistical Analysis

The data collected from the present study were tabulated in MS Excel and graphs were made in Origin 5.0 software. Descriptive statistics (i.e. ANOVA) and inferential statistics were applied to the collected data and the results were presented accordingly.

3. RESULTS

The results of both social survey (personal interview, questionnaire survey and personal observation) and water quality analysis obtained to know the impacts of "WASH intervention" implemented by IRSP in the targeted schools.

3.1. Sanitation Facilities

Latrine facility was the main problem in most schools, even each school had either one or hardly two latrines for huge of number of children. For instance, in one of the school with the strength of 70 students and two teachers only one latrine was available, which most of the time reserved for teachers and guests. However, the concern officials of IRSP reiterated that in the second phase two more latrines are planned to be constructed and that can be only reversed for the students. Still question a raised that how the two latrines can facilitate 70 students? As per guideline, there should be one latrine for every 25 students in girls' school and 30 students in boys' schools [3]. Also, in some schools in the targeted UCs few numbers of dysfunctional latrines were available, which had been constructed by the Government at the time of school's construction. In Figure 2, we calculated the number functional latrines in each UCs, which demonstrated an alarming low number of functional latrines as per school's requirements.

3.2. Hygiene Conditions of Targeted Schools

Environmental hygiene conditions and latrine cleanliness were another important issue, which were highlighted and extensively studied in the present study. Hygiene conditions of the schools in UC Takkar, Saroshah and Naray were observed to be highly alarming; wrappers were found in school's grounds and vicinity areas due to non-availability of dustbins or poor solid waste management mechanism. Other targeted areas have similar situation. However, schools in UC Madhay Baba were well maintained, classes were neat and clean, children were found using dustbins for throwing wrappers or plastics. Personal observation and social survey results revealed that existing sanitation facilities (i.e. latrines) were not maintained well in any of the UCs' targeted schools except Madhay Baba. Latrines usually produced bad smell, if they are not properly washed and maintained on daily basis. During the field visits and even in social survey, questions regarding latrine's cleanliness were asked. On site observations were done at different timings of schools showed that the situation of the latrine remained unhygienic throughout the day as they were hardly cleaned once in a day while few teachers shared their views through questionnaire that the latrine cleanliness activities were carried out on monthly basis in true spirit. However, the children had different opinions and they reflected that the latrine usually cleaned on daily basis, but their usages were so huge and cannot be seen clean every time in a day. Figure 3 shows the reply of the respondents about the cleanliness of latrines on daily basis in all the targeted UCs.

In addition, social survey and personal observation about the availability of dustbins facility showed that 50% schools in UC Takkar, Madhay Baba and Saroshah had classroom dustbins, while 80% schools of UC Naray was found to have dustbins within the classrooms. Environmental hygiene situation of schools in UC Naray and Madhay Baba was much better than schools in UC Takkar and Saroshah, which showed teachers as well as school management priority. Almost 80%

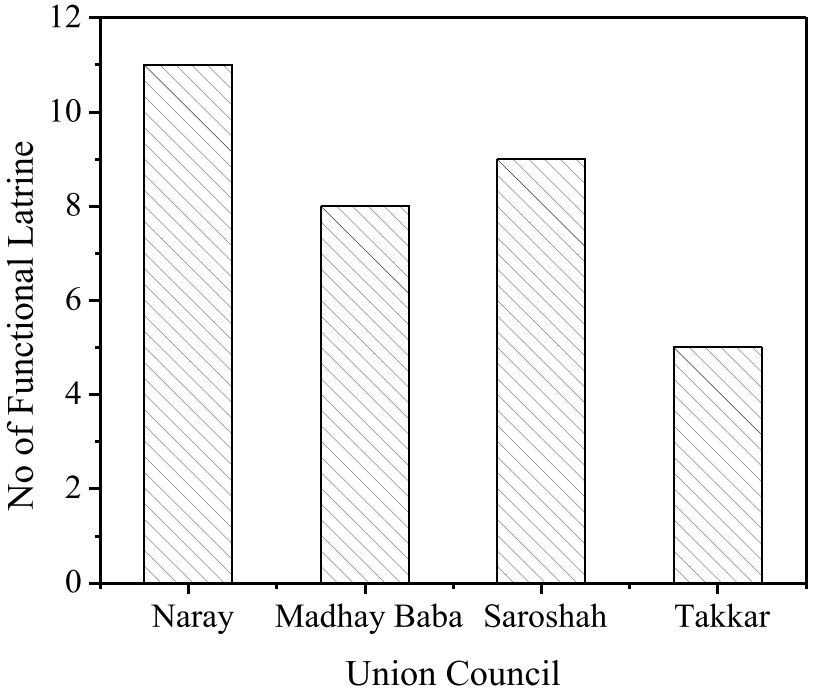

Fig. 2. No of functional latrines in each UC's

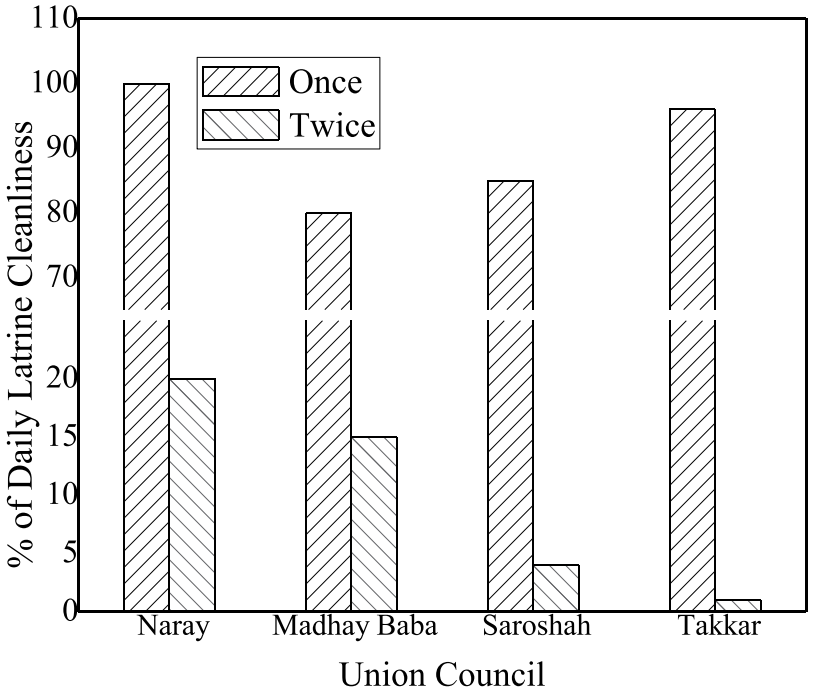

Fig. 3. Percentage of daily latrine's cleanliness (Once or Twice) in targeted UCs

of targeted schools in studied UCs were observed for children cleanliness monitoring in the morning assembly (gathering) and the data collected during FDGs showed that 85% of all the schools were taught hygiene lessons on daily basis.

3.3. Water Availability

Water availability in the washroom was a major issue in most of the targeted schools. All the targeted schools did not have proper water facility in their

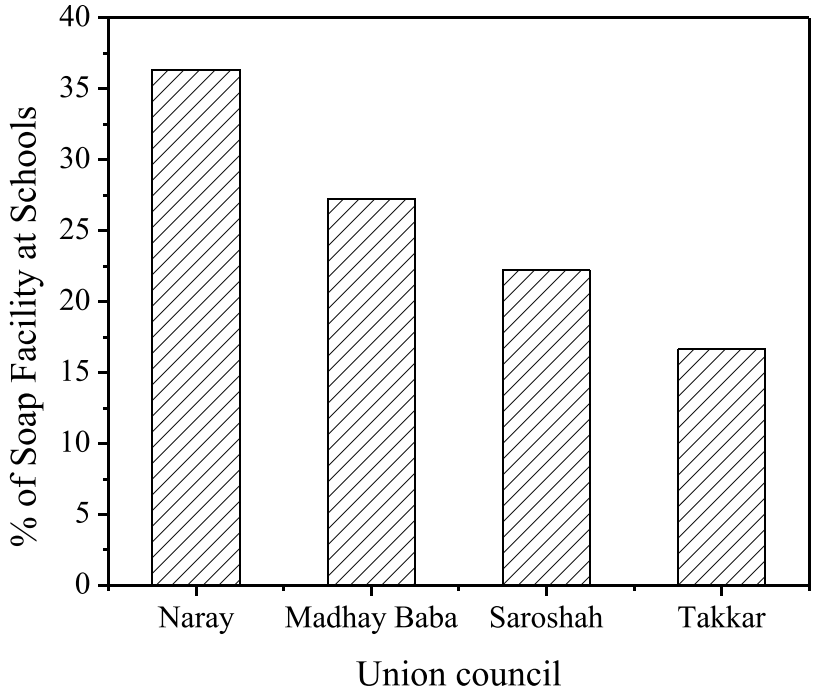

Fig. 4. Shows the % age of soap availability in different UCs for the users

latrines. Thus, the students were reluctant in using latrine due the non-availability of water for latrine flushing. Also, the available latrine cleanliness was one of the major issues in providing enabling environment for the students to use the latrines. In some cases, students fetch water from nearby homes or other places to fulfill the call of nature, especially for the girls students it was a major problem, which often forced them not to go schools and remain absent during nature cycle days.

3.4. Hand Washing Facility

Hand washing facility did not exist in any of the school in targeted UC neither hand washing was practiced by the students after latrine usage. No wash basin existed in any of the latrines in targeted UC, where children could wash their hands. Another important finding was the non-availability of soaps in the school latrines which was the main cause of not washing hands by the students after attending the latrines. Some of the schools in targeted UCs were reported to have soap facility for teachers only. Figure 4 shows the percentage of soap availability in different UCs for the users. Results demonstrated that UC Naray has high number of hand washing facility as compared to other UCs and which followed the order of Naray < Madhay Baba < Saroshah < Takkar. The reason of lowest hand washing facility in UC Takkar could be the result of lower number of WASH intervention in this UC as compared to other areas. Another reason could be the variations in WASH implementation by Government and NGOs, and also the old or new construction approaches, where the new construction requirements need such facility should be installed near the latrine.

3.5. Absentees and Drop out Ratio

There is a close linkage between the availability of WASH facilities in schools and students absenteeism. The school heads in many schools did not allow to take photos of their daily attendance registrar and restricted to only get verbal inquiry, which showed that many girls students in upper middle class left school due to the absent of latrine facilities and again many remained absent due to the non-availability of WASH facilities in schools. However, in some schools especially primary schools the level of schools attendance was quite satisfactory. The FGDs and personal interviews with the key respondents also revealed that there is close link between students' absenteeism and the availability of WASH facility. Many of the growing students reiterated that they will leave schools if the facility will not be installed and available to them and they may not go other vicinity homes to attend

latrines and call of nature.

3.6. Common Health Problems

In Pakistan, the numbers of people affected by the water borne diseases are in huge number, besides being on the track to achieve the Millennium Development Goals (MDGs) [3]. Many studies [15, 16] revealed the association of higher of diarrheal diseases and the poor water quality. In the present study, questionnaire survey, personal interview and the local BHUs (Basic Health Units) visits to get the daily OPD (Out Patient Department) list to know the number of daily patients with different diseases. The obtained results revealed the prevalence of higher number of diarrheal cases in all the targeted schools, as shown in Figure 5. Thus, the results showed that diarrhea was most common among the students of all the targeted UCs then other diseases followed by fever, flu and headache (Figure 5). Among the targeted UCs, UC Naray was higher number of diarrheal cases and the reason could be the non-availability of enough number of WASH facilities in schools. Also, hygiene practices of the students were not up to the mark and the basic facilities for keeping good hygiene environment was not satisfactory.

3.7. Water Quality Results

The assessment of drinking water quality by analyzing some of the selected water quality parameters is important to know the overall water quality conditions in the targeted schools. In general, the provision of clean drinking water to the children can help them to fight against the pathogenic water borne diseases. In the present study, water samples were collected from all the schools' tap stand and tested for different parameters such as pH, turbidity, electrical conductivity (EC), total dissolved solids (TDS), E. coli and total coliforms in IRSP Lab, Mardan. The results obtained after analyzing the water samples collected from different schools were used for statistical analysis using descriptive ANOVA. Results revealed that all the samples had no E. coli or other harmful bacteria resultantly the E. coli results were not mentioned in Table 1 and subsequently analyzed using statistics like descriptive ANOVA test. There was no treatment facility for drinking water available and drinking water provided from the local source was safe for drinking. The results showed that all the samples collected from different schools had no E. coli and each samples showed different number of total coliforms.

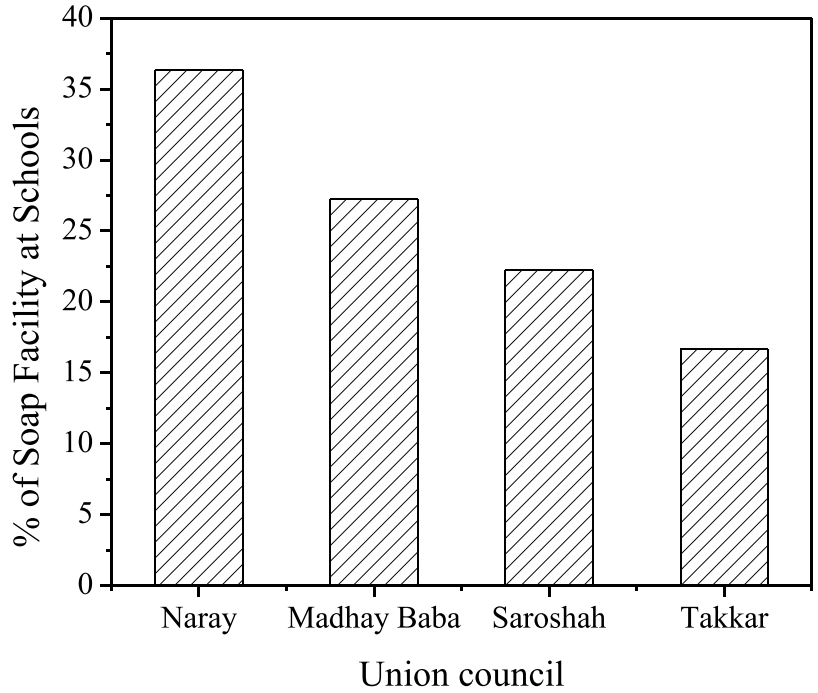

Fig. 5. Presents the %age of children suffering in the top four common diseases in the study area

Table 1. ANOVA test results of the selected water quality parameters

ANOVA		Sum of Squares	df[a]	Mean Square	F[b]	Significance
pH	Between Groups	1.118	12	0.093	1.108	0.395
	Within Groups	2.187	26	0.084		
	Total	3.306	38			
Turbidity (NTU)	Between Groups	6.296	12	0.525	6.042	**0.000**[c]
	Within Groups	2.258	26	0.087		
	Total	8.554	38			
Electric Conductivity (µS/cm)	Between Groups	7287051.897	12	607254.325	571.278	**0.000**
	Within Groups	27637.333	26	1062.974		
	Total	7314689.231	38			
TDS (mg/L)	Between Groups	3114902.103	12	259575.175	666.937	**0.000**
	Within Groups	10119.333	26	389.205		
	Total	3125021.436	38			
Total Coliform (CFU/100 mL)	Between Groups	140083.641	12	11673.637	119.275	**0.000**
	Within Groups	2544.667	26	97.872		
	Total	142628.308	38			

The mean difference is significant at a level of 0.05.
[a] Degree of freedom
[b] F-statistic
[c] Bold values are significant

Table 1 presents the results analyzed using ANOVA test of the selected parameters. The water quality results after descriptive ANOVA analysis revealed that all the parameters were significantly varied ($P<0.05$), except the water pH values. It can be seen from the descriptive ANOVA table that pH value has a p value greater than 5%, which showed that pH has no such effect, while the remaining other parameters showed that there was a significant effect or they have varied effects.

4. DISCUSSION

The provision of clean drinking water is a basic human right and according to the Pakistan National Drinking Policy of 2009, its supply to the entire Nation comes under the responsibility of the state [16-18]. Pakistan, like many other developing countries is plagued by in sufficient provision of sanitation facilities and dysfunctional water supply systems. However, surprisingly the latest figures provided by the Joint Monitoring Report [3, 19] reveals that Pakistan has met the target of attaining improved sanitation and is making well progress in achieving safe drinking water supplies. However, the personal observation in every corner of the country presented completely different scenarios and water quality and environmental sanitation conditions are quite worse and increasing number water borne diseases have been reported across the country [16]. In the few decades, international donor agencies including UNICEF invested billions of dollars to provide the WASH facilities in order to meet the Sustainable Development Goals (SDGs) or agenda 2030. In the light of such donor intervention, the present initiative taken up by IRSP (a local NGO in Mardan district) from donor support to implement WASH programme at Takhtbhai, which was no doubt a positive step and different schools in Takhtbhai were surveyed and the provision of WASH programme related activities were undertaken. The present study was carried out in

order to evaluate the impact of WASH programme in schools implemented by IRSP to promote the health and hygiene conditions of rural students in district Mardan. The results for different schools in the targeted council were obtained through different methodologies and measured the effectiveness of the project activities. An international research organization (i.e. Center for Affordable Water and Sanitation Technology (CAWST)) developed methodology for evaluating education activities [20], which has been implemented and replicated in many other countries. However, in Pakistan no such follow up or performance evaluation was carried out for WASH activities carried out in different organizations.

The sanitation facility for students in each UC was not sufficient for students and even the teachers and did not even meet WHO standards (1 toilet per 25 girls and 1 toilet per 30 boys) [3, 13]. Therefore, teachers and students had to share the same latrine some times while in other schools students had to wait for their turns for longer time. Latrine cleanliness was another important issue which was common among the schools of all the targeted UCs, because majority of the students were not aware of sanitation or hygiene education neither the type of sanitation facilities existed in their schools was improved. The students in primary schools were not trained for proper using of latrines; therefore it was found that education gap existed regarding WASH programme different components, which could be overcome by more focusing on the hygiene education component and awareness rising. For such activities participatory involvement of all the students can bring positive changes in students' attitude towards hygiene education and development [21]. Previous studies carried out on WASH impact on absentees and drop out ratio among school going children had shown significant effect unlike the present study. The existing poor WASH conditions in different targeted schools had impact on the child attendance or drop out ratio through the examination of children attendance registers. Therefore, absentees and drop out ratio among the children of targeted schools was too low and no such impact on attendance or drop out was recorded. However, Tarrass and co-workers [22] reported that many low income countries lack basic WASH facilities, which affect school going children's health and performance and subsequently increase the schools drop out ratio.

Hygiene practices by the students in the targeted schools was very rare such as availability and use of class room dustbins, use of soaps for washing hands was not common in all the schools though 80% targeted school children were given hygiene lesson on weekly basis while 85% of the targeted school were reported to have cleanliness monitoring of children in morning on daily basis. The access to inadequate WASH facilities causes nearly 1.5 million deaths of children under the age of five and also leads to over 272 million days loss due to diarrhea [23]. Similarly, in the present study it was found that children of Takhtbhai were more likely affected by diarrhea than other common health problems such as fever, flu and headache. Drinking water collected for the analysis of different parameters recorded that no E. coli was detected in any of the sample collected and was according to the standards set up by WHO and Pakistan National Environmental Quality Standards [18], which means that the water had no bacterial contamination while diarrhea was still common among the targeted school which may be due to their unhygienic conditions in schools, lack of healthy hygiene practices by the students and non-availability of soaps for washing hands. In social survey and interaction, children of different schools also came up with very good suggestions which could bring positive change to the school environment. Children from different schools during FGDs were able to openly share their views and problems related to hygiene due to the absences of different facilities at schools. They even shared their learning experiences related to hygiene lessons taught at schools and gave their suggestions as well in order to overcome problems related to WASH facilities at school.

5. CONCLUSIONS

This study was carried out to evaluate the impacts of WASH programme on student's health and hygiene conditions in different targeted schools in selected UCs of Takhtbhai, district Mardan. Results of social study revealed that none of the schools in UC Takkar and Saroshah followed up WASH programme implemented at their schools, neither the children were encouraged to take the initiative for improving the hygiene conditions of their schools. The data collected after testing the drinking water samples for physical and chemical parameters

was evaluated through descriptive statistical analysis using ANOVA, which revealed that all the parameters showed significant variations and affect each other except pH. While water samples tested for bacteriological analysis showed that none of the water sample collected from the targeted schools in the targeted schools of all UCs, which showed that the bacteriologically water quality is safe for human consumption. Findings from the present study suggested that the implementation of WASH programme with true its spirit can bring positive changes in schools going children.

6. ACKNOWLEDGEMENTS

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Research Article

Effect of Papain Enzyme in Feed on Digestibility of Feed, Growth Performance, and Survival Rate in Post Larvaes of Freshwater Lobster [*Cherax quadricarinatus* (Von Martens, 1868)]

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Abstract: The study aimed to find the impact of additional papain enzyme in feed digestibility, growth performance, and survival rate in post larvaes of freshwater lobster [*Cherax quadricarinatus* (Von Martens, 1868)]. There were five treatments with dosage A (0 % papain enzyme per kg feed), B (0.1 % papain enzyme per kg feed), C (0.2 % papain enzyme per kg feed), D (0.3 % papain enzyme per kg feed) and E (0.4 % papain enzyme per kg feed). Every treatment was repeated three times. The experiment used Completely Randomized Design. EFU (Efficiency of Feed Utilization), FCR (Feed Conversion Ratio), PER (Protein Efficiency Ratio), RGR (Relative Growth Rate), ADCP (Apparent Digestibility Coefficient of Protein), SR (Survival Rate) and water quality parameters were determined. The study found that the impacts of the various dosages of papain enzyme exhibited significant effects ($P < 0.01$) on the EFU, FCR, PER, RGR, ADCP; however, insignificant effects were observed ($P > 0.05$) on the survival rate of freshwater lobster. It was concluded that the incorporation of papain enzyme improved the feed digestibility and the growth performance of post larvae in freshwater lobster. The study found that optimum dosages of papain enzyme on feed digestibility, efficiency of feed utilization and growth rate of freshwater lobster ranged from 0.24 % to 0.31 % papain enzyme per kg feed. The quality of media culture was still in the feasible condition for post larvae of freshwater lobster cultivation.

Keywords: Artificial feed, Diet management, Diet efficiency, Nutrient absorption, Proteolytic enzyme.

1. INTRODUCTION

Freshwater lobsters [*Cherax quadricarinatus* (Von Martens, 1868)] have high economic value in food consumption and decorative purposes. Farming of freshwater lobsters highly depends upon the supply of feed. Akiyama et al. [1] reported that the cost portion of diet for fish cultivation is around 60 % of total cost whether it is reared in semi-intensive or intensive technology. The quality of artificial feed is determined by nutrient composistion, nutrient balance and utilization efficiency. Therefore, better diet management is needed to increase diet utilization efficiency. The efficiency could be boosted by adding enzyme in diet [2] and digestion efficiency of the cultured species can be increased by supplementing the enzymes to the feed [3]. The addition of enzymes breaks down protein into shorter peptides that speeds up the digestion of protein [3–6] favoring the feed utilization. One of the many enzymes used in diet fish industry is papain. Papain is categorized as protease enzyme

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that can hydrolyze protein into short peptide. It is an important factor to boost digestibility of protein and absorption and also to enhance growth [7]. Panigrahi et al. [8] stated that papain enzyme is proteolytic enzyme that can hydrolyze protein into amino acid or peptides. Papain enzyme contains 187 amino acids weighing 21 000 moles. It has sulfhydryl function compound and is able to hydrolyze peptide compound into lysine and glycine amino acids [9]. However, until recently there has been lack of study of papain enzyme incorporated feed on feed digestibility, efficiency of feed utilization and growth rate of freshwater lobster for post larvae of freshwater lobster (*C. quadricarinatus*). Morerever, reference [3, 10–12] suggested that the papain enzyme addition in the diet would help and accelerate nutrient absorption; in turn it can provide enough nutrients for fish growth and survival.

2. MATERIALS AND METHODS

2.1. Animal Test

Animal test used in the study was post larvae of freshwater lobster (*C. quadricarinatus*) with initial body weight averaging (0.43 ± 0.09) g. As many as 150 post larvaes were collected from the Center for Freshwater and Brackish Aquaculture, Jepara, Central Java, Indonesia. They were first acclimated for one week in the 500 L fiber container in order to be able to adjust to the environment and the diet. During acclimatization the post larvaes have been fed with control diet [10]. To avoid stress on the larvaes aeration was installed. Feces and diet waste were discarded by syphoning 25 % water every day and then refill with fresh water.

2.2. Diet Test

Diet test used in the study was in the form of pellets containing 30 % protein [13]. Various doses of papain enzyme [(0; 0.1; 0.2; 0.3; and 0.4) % kg^{-1} feed] were added into the artificial diet. This experiment was modified from [10] that they used papain enzyme with dose of 0.1 % kg^{-1} feed in the post larvae of *M. rosenbergii*. The composed diet consisted of fish meal, soybean meal, corn meal, rice bran, wheat flour, fish oil, corn oil, mineral mix and Cr_2O_3 0.5 % [14] and papain enzyme. The brand of

Table 1. Ingredients (%) and proximate composition (% of dried mass) of the artificial diet for post larvae of *C. quadricarinatus*

Ingredients	**Composition (%)**				
Diet Composition	**A**	**B**	**C**	**D**	**E**
Papain	0	0.1	0.2	0.3	0.4
Fish meal	27.5	27.5	27.5	27.5	27.5
Soybean meal	23.8	23.8	23.8	23.8	23.8
Corn meal	19.3	19.3	19.3	19.3	19.3
Rice bran	19.7	19.7	19.7	19.7	19.7
Wheat flour	4.7	4.6	4.5	4.4	4.3
Fish oil	1.0	1.0	1.0	1.0	1.0
Corn oil	0.5	0.5	0.5	0.5	0.5
Vit Min Mix	2.0	2.0	2.0	2.0	2.0
Cr_2O_3	0.5	0.5	0.5	0.5	0.5
CMC	1.0	1.0	1.0	1.0	1.0
Total	100	100	100	100	100
Proximate Analysis Results					
Protein (%)*	30.04	30.02	30.05	30.15	30.06
Fat (%)*	7.87	7.97	7.84	7.63	7.49
BETN (%)*	36.54	36.51	36.55	37.24	37.68
Energy (kcal) *	260.67	260.82	260.01	260.44	260.06
Ratio E/D (kcal g^{-1} Diet)	8.67	8.69	8.65	8.64	8.65

Notes:

a. The values were calculated based Digestible Energy [27] for 1 g protein equals 3.5 kcal, 1 g fat equals 8.1 kcal, and 1 g carbohydrate equals 2.5 kcal.
b. According [28], the optimal E/P ratio for growth ranges from 8 kcal g–1 to 12 kcal g–1.
c. *Animal Nutrient Laboratory, Faculty of Husbandry and Agriculture, Diponegoro University (2017).

papain enzyme used in the study was "NEWZIME" produced by the Center for Freshwater and Brackish Aquaculture, Jepara, Central Java, Indonesia.

2.3. Elements and Proximate Composition

Mixing the diet was done in order. First was to mix the diet ingredients with the smallest amount and final step was to mix the diet ingredients with the largest amount, except fat ingredients (fish oil and corn oil) added after all ingredients mixed. The ingredients were mixed homogenously then they were manufactured into 1 mm to 2 mm pellets. The pellets were dried in the oven with the temperature ± 40 °C [14]. The diet elements and Proximate Composition is shown in the Table 1. Nitrogen content was measured using Microjedhal method [15], while protein content was measured by multiplying nitrogen with 6.25 constant. Soxhlet extraction method with petroleum ether was used to analyze raw fat content [15]. Ash content was obtained by burning the diet at 550 °C in the furnace for 5 h [15]. Carbohydrate was measured using Remainder method [16].

2.4. Experiment Procedure

There were five treatments which were by adding different doses of papain enzyme. Every treatment was repeated three times. The experiment used Completely Randomized Design. The larvaes were raised in circular plastic containers with the capacity of 30 L each. There were 15 containers and each was filled with 30 L of water. Each container had 10 larvaes of freshwater lobster. To maintain the containers cleaned, the fish feces and the feeding waste were discarded by syphoning 25 % water every day and then refill with fresh water. Feeding time was three times a day at 11:00, 15:00, and 19:00. The larvaes were given diet at 5 % of the mass weight [13]. Since freshwater lobsters are nocturnal, feeding during the night was given more compared to during the day. At the beginning of the study the larvaes were scaled. To evaluate growth the sampled larvaes were scaled every week for 42 d. All data of experiment were recorded [10]. The measurement of water quality was conducted in the morning at 8:00 and in the afternoon at 17:00. It used Water Quality Checker to measure dissolved oxygen and acidity of the media. Thermometer was also used to measure the temperature of the media. Measurement of ammoniac was done at the beginning and at the end of the study.

Parameters used in this study were adopted from Tacon et al. [17]. There were Efficiency of Feed Utilization (EFU), Feed Conversion Ratio (FCR) and Protein Efficiency Ratio (PER), Relative Growth Rate (RGR), while apparent digestibility coefficient of protein (ADC_P) was adopted from Gul et al. [4], and Survival Rate (SR) was adopted from National Research Council [14]. The modified colorimetric method was used to analyze chromic oxide level in the feed and feces [4]. The values were gauged with a spectrophotometer (540 nm) (Shimadzu UV-2102 PC, UV-visible Scanning Spectrophotometer). After per chloric acid oxidation and a colored complex with diphenyl carbazide (DPC) have been formed, the measurement was done. pH

$$\text{EFU}: \frac{(\text{Final weight} - \text{Initial weight})}{\text{The amount of feed consumed}} \times 100\ \% \quad (1)$$

$$\text{FCR}: \frac{\text{The amount of feed consumed}}{(\text{Final weight} + \text{Total weight fish death}) - \text{Initial weight}} \times 100\ \% \quad (2)$$

$$\text{PER}: \frac{\text{Final weight} - \text{Initial weight}}{\text{The amount of feed consumed} \times \text{Protein content of feed}} \times 100\ \% \quad (3)$$

$$\text{RGR}: \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight} \times \text{Time experiment}} \times 100\ \% \quad (4)$$

$$\text{ADC}_P: 100\left\{\frac{\%\ Cr_2O_3 \text{ in the feed}}{\%\ Cr_2O_3 \text{ in the feces}} \times \frac{\%\ \text{protein in the feces}}{\%\ \text{protein in the feed}}\right\} \quad (5)$$

$$\text{SR}: \frac{\text{Final count}}{\text{Initial count}} \times 100\ \% \quad (6)$$

(Jenway 3510), DO (Jenway 970), temperature and ammoniac (HANNA: HI. 8633) were tested for water quality variables. Each container has an aerator to recirculate the water. The variables were measured by Equation (1) to Equation (6) :

2.5. Statistical Analysis

Before analyzing ANOVA, normality, additivity, and homogeneity tests were conducted to guarantee that the data have normal, homogeneity and additive properties. If the probability results of ANOVA s are $P < 0.05$ or $P < 0.01$, it requires Duncan Test. Polynomial Orthogonal Test with SAS9 and Maple12 [18] were used to measure papain enzyme optimal dose, while water quality analysis was thoroughlyexplained.

3. RESULTS AND DISCUSSION

Data on EFU (Efficiency of Feed Utilization), FCR (Feed Conversion Ratio), PER (Protein Efficiency Ratio), RGR (Relative Growth Rate), ADC_P (Apparent Digestibility Coefficient of Protein), SR (Survival Rate) of *C. quadricarinatus* post larvaes were presented in the Table 2.

Until now, there is no any research on additional papain enzyme in the diet for *postlarvae C. quadricarinatus*, however, there were some studies on the effect of photolytic enzyme for other species, such as study by Sajjadi and Carter [19] reported that *Salmo salar* (Linnaeus, 1758) has better growth due to additional protolithic enzyme in the diet. Singh et al. [3] reported that the feeding fish with the 2 % papain addition has the lowest ratio of feed conversion, higher growth rate, digestibility of protein, and a ratio of protein efficiency of *Chanos chanos* (Forsskål, 1775). Patil and Singh [10] stated that the results of this feeding trial show that the 0.1 % papain enzyme incorporated diet for post-larvae of *Macrobrachium rosenbergii* (De Man, 1879) has better growth and feed utilization. Khati et al. [11] stated that papain enzyme was able to increase digestibility of nutrient and also to improve the health of *Labeo rohita* ((Hamilton, 1822). Muchlisin et al. [12] reported that the papain enzyme optimum dosage of for keureling fish [Tor tambra (Valenciennes, 1842)] was 27.5 mg kg–1 feed.

The addition of papain enzyme in the diet successfully improved the efficiency of feed utilization of postlarvae of *C. quadricarinatus*. It was indicated by the higher feed efficiency. Gatlin [20] reported that nutrient efficiency is an indicator in nutrient utilization by the fish in which low nutrient efficiency ratio show that the nutrient has been digested and optimally absorbed by the fish. The efficiency of feed utilization is the ratio between the growth of body weight and feed during cultivation [17].

ANOVA test shows that the impacts of papain enzyme addition in the feed was significant ($P < 0.05$) on Efficiency of Feed Utilization (EFU) of postlarvae of *C. quadricarinatus*. This study as shown in Table 2 that the D treatment (0.3 % kg–1 feed) had the highest EFU as much as 73.09 % and the lowest was A treatment (0 % papain enzyme per kg feed) as much as 60.82 %. The high EFU value in treatment D (0.3 % papain enzyme per kg feed) show that freshwater lobster only used little protein for regular activities; therefore, the rest was to support growth. It was indicated by the highest of RGR value of D treatment compared to treatments of A (8.48 % d–1), B (9.25 % d–1), C (9.89 % d–1) and E (9.33 % d–1). The high efficiency indicated that the diet has high quality, therefore it can be utilized efficiently [14]. Moreover, Singh et al. [3] also suggested in their studies that the enzyme could help fish to breakdown complex big organic molecules, such starch, cellulose and protein into simpler forms; therefore, it can increase diet efficiency. The higher the efficiency of feed utilization was higher was the growth.

Cubical equation between papain enzyme addition in the feed and EFU of postlarvae of *C. quadricarinatus* was $Y = -810.56x3 + 372.02x2 – 2.2127x + 61.22$ with $R2 = 0.88$. The equation was obtained from the orthogonal polynomial test (Figure 1). The papain enzyme optimum dose in the feed on the EFU was 0.31 % kg–1 feed with EFU maximum of 73.51 %.

The effect of papain enzyme addition was significant ($P < 0.05$) on the ratio of feed conversion (FCR) in the post-larvae of *C. quadricarinatus* (Tabel 2). The value of the ratio of feed conversion was better as the dose of the enzyme increased [(0.1 to 0.3) % papain enzyme per kg feed]. Papain enzyme implementation has resulted in an increase in the ratio of feed conversion. It was because of better metabolism in fish fed on papain enzyme

Table 2. Efficiency of Feed Utilization (EFU), Feed Conversion Ratio (FCR), Protein Efficiency Ratio (PER), Relative Growth Rate (RGR), Apparent Digestibility Content Protein (ADCP) and Survival Rate (SR) of *C. quadricarinatus post larvae*

Experiment Data	Treatments				
	A	B	C	D	E
EFU (%)	60.82 ± 0.54^{c}	65.96 ± 0.97^{b}	67.65 ± 0.57^{b}	75.09 ± 0.65^{a}	69.35 ± 0.36^{b}
FCR	2.55 ± 0.10^{c}	2.46 ± 0.24^{b}	2.26 ± 0.24^{b}	1.76 ± 0.07^{a}	2.30 ± 0.22^{b}
PER	1,90 ± 0.04^{c}	2.19 ± 0.23^{b}	2.389 ± 0.23^{b}	3.75 ± 0.05^{a}	2.24 ± 0.25^{b}
RGR (% d^{-1})	8.48 ± 0.20^{c}	9.26 ± 0.15^{b}	9.89 ± 0.20^{b}	11.01 ± 0.23^{a}	9.33 ± 0.24^{b}
ADC_P	60.47 ± 0.02^{c}	78.65 ± 0.05^{b}	79.15 ± 0.05^{b}	83.93 ± 0.05^{a}	77.65 ± 0.04^{b}
SR (%)	93.33 ± 5.77^{a}	90.00 ± 10.0^{a}	90.00 ± 10.0^{a}	96.67 ± 5.77^{a}	93.33 ± 5.77^{a}

Note: The values with the same superscripts in the column show that there was no difference

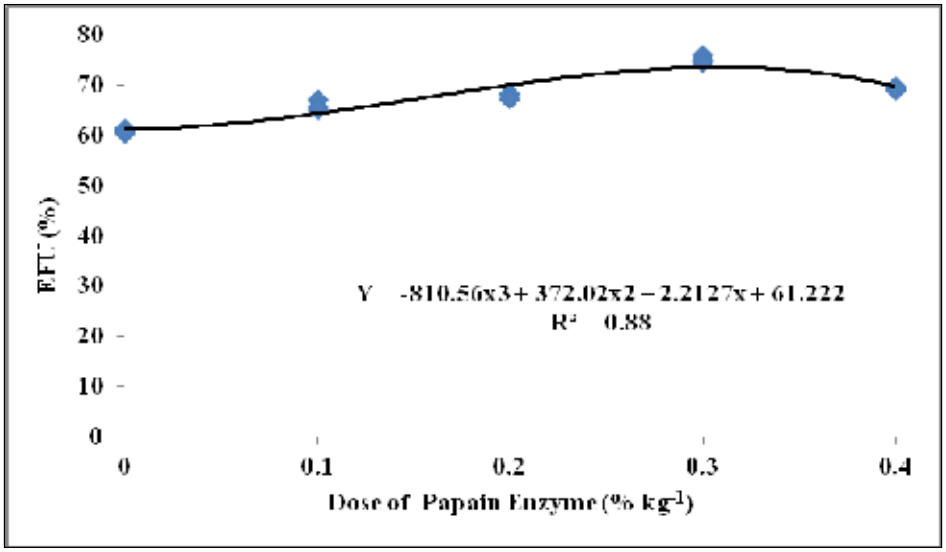

Fig. 1. Graph of the relationship between papain enzyme addition in the feed and EFU of post larvae of *C. quadricarinatus*

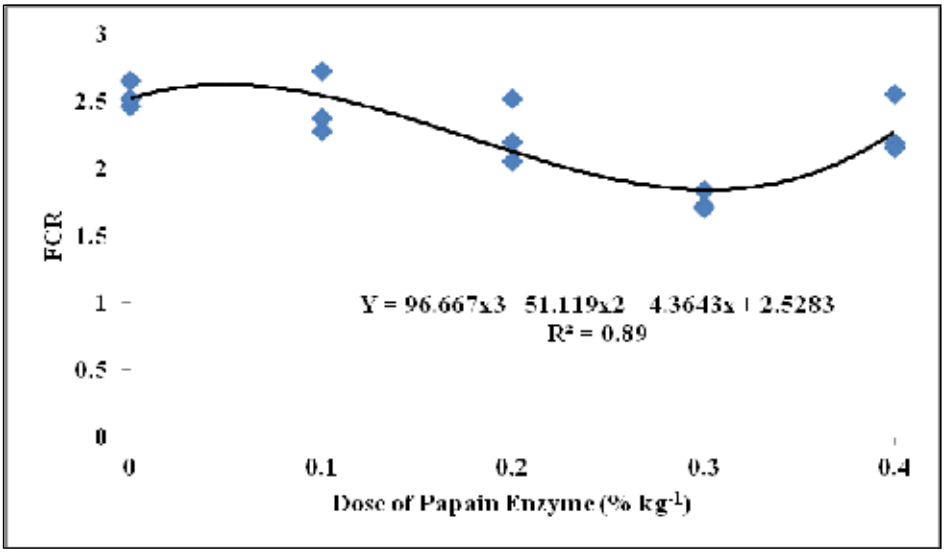

Fig. 2. Graph of the equation between papain enzyme incorporated feed and FCR of post larvae of *C. quadricarinatus*

incorporated diet. In turn, it has better FCR. The lowest FCR with the value of 1.75 was obtained in treatment D in which the dose of the papain enzyme was 0.3 % kg–1 feed and followed by the treatments of C (2.26), E (2.30), B (2.46) and A (2.55). It could be concluded that the papain enzyme addition could improve the feed utilization efficiency and reduce a ratio of feed conversion, as results of the study that treatment D brought about the highest EFU (75.09 %) and the lowest FCR (1.75). Similar results were reported by reference [11, 12, 21].

The cubical equation on the relationship of papain enzyme in the feed and the FCR of Y = 96.667x3 - 51.119x2 + 4.3643x + 2.5283 with R2 = 0.89 was obtained from the orthogonal polynomial test (Figure 2). The papain enzyme optimum dose in the feed on the FCR was 0.31 % per kg feed with FCR maximum of 1.84.

The ratio of Protein Efficiency Ratio (PER) indicates protein sources in diet that provide essential amino acids required by fish [11]. The study showed that a ratio of protein efficiency has increased in all PER treatments that ranged from 2.19 to 3.75 (Table 2). It was always higher than the treatment without papain enzyme. Protein efficiency ratio is the measurement to indicate how good protein in the diet can provide essential amino acids for fish [22]. The highest ratio of protein efficiency was obtained in the D treatment (0.3 % kg–1 feed). It was expected that amino acid composition in the treatment D (0.3 % kg–1 diet) was suitable with the amino acid in the body of postlarvae of *C. quadricarinatus.* Similar results were also reported by reference [3, 11, 12].

Cubical equation between papain enzyme in the feed and the PER was Y = -231.94x3 + 121.74x2 – 10.742x + 1.9791 with R2 = 0.80. ThisThe equation was obtained from the test of the orthogonal polynomial (Figure 3). The papain enzyme optimum dosage in the feed for the PER was 0.29 % papain enzyme per kg feed and the value of PER was 1.84.

The results of variance analysis displayed significant impact ($P < 0.05$) with incorporating papain enzyme ion relative growth rate (RGR) of freshwater lobster *(C. quadricarinatus)*. The lowest RGR was on the treatment A (0 % kg–1 feed). It was suspected that there was no papain enzyme which hydrolyzed polypeptide in the diet. It caused low in diet digestibility and efficiency, in turn, it hindered freshwater lobster growth. The results show that treatment A generated low RGR as well as ADCP and EFU. The highest RGR was obtained in the D treatment (0.3 % papain enzyme per kg feed). It can be concluded that the addition of 0.3 % papain enzyme on every kg feed was the optimal dosage. The papain enzyme was to hydrolyze protein into short chain peptide in order to increase protein digestibility, nutrient efficiency, and growth of freshwater lobster. The results show that treatment D generated high RGR as well as ADCP and EFU. The result was the same as the study conducted by Mo et al. [21]. He found that papain is the protease enzyme that hydrolyzes protein into short chain peptide in the feed. It is the main factor in increasing protein digestibility, fast absorption, and growth. Moreover, Miyamoto et al. [23] stated that papain enzyme can hydrolyze lipid, carbohydrate, and protein. An exogenous enzyme has proved that it can increase feed nutrients as reported by reference [3, 10–12].

Cubical equation between papain enzyme in the feed and the RGR was Y = -220.28x3 + 100.55x2 – 2.8496x + 8.5393 with R2 = 0.90. The equation was derived from the test of an orthogonal polynomial (Figure 4). The papain enzyme optimum dose in the feed for the RGR was 0.29 % per kg feed. The maximum value of RGR was 10.78 %.

Table 2 show that the addition of papain enzyme (0.1 to 0.4) % per kg feed could boost the apparent digestibility coefficient of protein (ADCP). It was because of extensive protein hydrolysis that was induced by papain [11]. The highest coefficient of protein digestibility was obtained in the D treatment (0.3 % per kg feed). The value was 83.93 %. The papain dose was suitable that could increase the protein breakdown, protein digestibility, protein absorption and produce short peptides in diet, which in turn it increased growth. Dawood et al. [24] thought that the addition of proteolytic enzyme would improve protein content. Moreover, Singh et al. [3] found that the addition of papain enzyme into the feed can boost protein digestibility. Similar results were reported in the studies of *C. chanos* [3], *L .rohita* [11], and *T. tambra* [12].

Cubical equation between papain enzyme in the feed and the ADCP was Y = -551.94x3 - 650.07x2

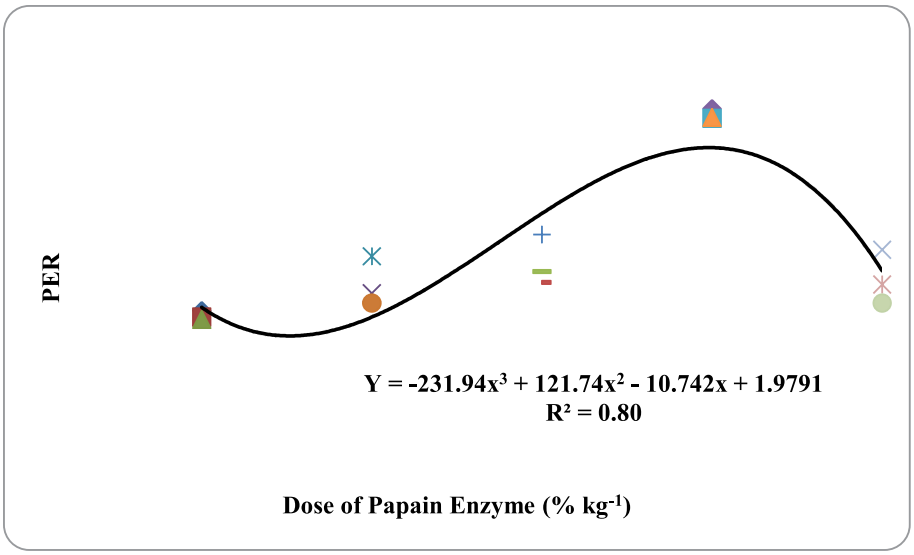

Fig. 3. Graph of the equation between papain enzyme incorporated feed and PER of post larvae of *C. quadricarinatus*

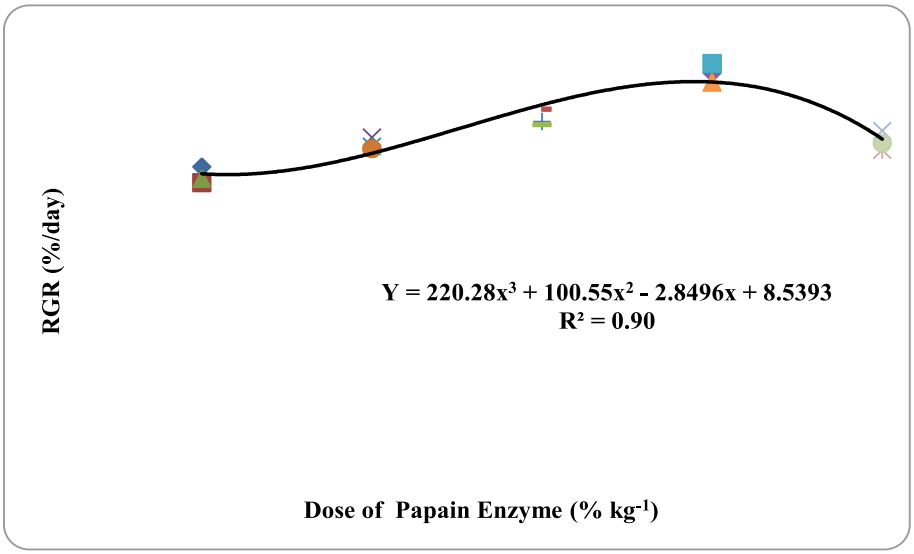

Fig. 4. Graph of the relationship between papain enzyme in the feed and RGR of post larvae of *C. quadricarinatus*

+ 214.68x + 61.003 with R2 = 0.94. The equation was derived from the test orthogonal polynomial (Figure 5). The papain enzyme optimum dose in the feed on the ADCP was 0.24 % per kg feed with ADCP maximum of 82.72 %.

The variance analysis results show that the effect of papain enzyme addition was not significant ($P > 0.05$) on the survival rate (SR) of postlarvae of *C. quadricarinatus*. Yakuputiyage [25] informed that the diet did not affect the survival rate, because the survival rate was influenced by the initial treatment of the fish and water quality. Similar findings were declared in the studies of *C. chanos* [3], *M. rosenbergii* [10] and L. rohita [11]. Hepher [26] found that the survival rate was influenced by the gender of fish, heredity, age, reproductive characteristics, disease prone and external factors such as water quality, density, amino acids in the diet. Water quality was still on condition that overpassed to the cultivation of post larvae of *C. quadricarinatus*. The measurement of water

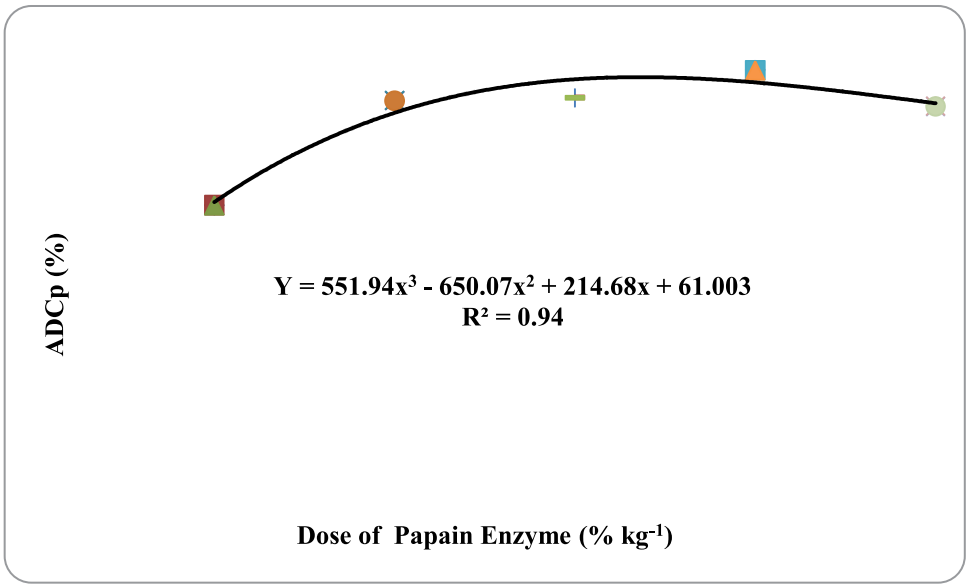

Fig. 5. Graph of the relationship between papain enzyme addition in the artificial feed and ADCP of post larvae of *C. quadricarinatus*

Table 3. Parameters of water quality for cultivation of Freshwater Lobster (*C. quadricarinatus*)

Variables	Values A	B	C	D	Feasibility
Temperature (°C)	25.1 to 28.2	25.1 to 28.2	25.2 to 28.2	25.1 to 28.1	25 to 32*
pH	8.63 to 8.86	8.64 to 8.77	8.70 to 8.79	8.66 to 8.82	7 to 9*
DO (mg L^{-1})	3.01 to 4.0	3.05 to 3.63	3.04 to 3.62	3.00 to 3.63	4 to 6*
Ammoniac (mg L^{-1})	0.25 to 0.6	0.25 to 0.6	0.25 to 0.6	0.25 to 0.6	< 1*

Note: *[29]

parameter during postlarvae of *C.quadricarinatus* cultivation was presented in Table 3.

4. CONCLUSION

The study concluded that the papain enzyme addition could boost the digestibility of feed and the growth performance of post larvae of freshwater lobster (*C. quadricarinatus)*. The optimum doses of papain enzyme on digestibility of feed and the growth performance of freshwater lobster (*C. quadricarinatus)* ranged from (0.24 to 0.31) % kg^{-1} feed.

5. ACKONWLEDGEMENTS

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Research Article

Exploitation of Polymorphic Sequences in Chloroplast Genome for Identification of Commercial Tobacco Cultivars

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Abstract: Tobacco is an important commercial commodity. Discrimination of tobacco cultivars at DNA level is extremely important for selling of brand, free of mixing. Chloroplast markers have sufficient resolving power for differentiation at intra-varietal level. In this study only chloroplast genome markers were used to amplify polymorphic regions encoding genes involved in photosynthetic machinery of plants. This region encompassing 9000 to 9600bp harbors SNPs, Indels and SSRs. Different bioinformatics and phylogenetic software were used to investigate relationship of DNA sequences of 11 unknown plant samples with a reference sequence from NCBI. Multiple alignments exhibited sequence conservation encompassing a substantial region. But it also revealed important variety specific SNPs. Sequence similarity index was generated for grouping of unknown plants. The unidentified plants were differentiated into different clusters as revealed by phylogenetic analysis. Furthermore, a neighbor-net network analysis validated the results of Neighbor Joining tree. Our analysis indicates that there exist at least 5 varieties of tobacco. The V1 is the most distant cultivar. V5 is also separated from the rest of cultivars. But V2, V3 and reference sequence make further linage, and can be considered as separate clade. A bigger cluster including V7, S2 and V6 is differentiated into distinct group. The S1, V9, V10 and V8 are the 5th cluster that can be considered as a single variety. Hence there were 5 varieties in total that were mixed in 11 samples.

Keywords: Tobacco, Varietal identification, Chloroplast markers, DNA fingerprinting

1. INTRODUCTION

Tobacco (*Nicotiana tabacum L.*) is one of the most significant commodities in agriculture. Across the world 33 million people depend on tobacco cultivation for their income (International Tobacco Growers Association (ITGA), 1996). The use of tobacco commercial varieties is important for brand selling. Mixing of elite varieties with ordinary may lead to non-acceptable taste and devalue the brand. This leads to economic losses. Therefore, use of pure and authentic varieties is inevitable for further propagation [1,2]. Reliable variety identification is much needed approach in many cultivars. Traditionally, this was achieved by utilizing morphological and biochemical markers but nowadays DNA markers have gained much popularity [3]. There are numerous benefits of employing molecular markers over other options. They are highly variable and co-dominantly inherited. They are easily visible and distributed uniformly over the entire genome. They are constant, economical and simple to use. Usually they need minor quantity of DNA and no prior information related to genome is required [4]. Recently, Yan et al. [5] has reviewed the applications and limitations of universal CpDNA markers in discriminating East Asian evergreen oaks. Sequencing of entire chloroplast genome has unveiled many polymorphic regions that can be selected for varietal identification through PCR amplification and eventually sequencing. These include SNPs, Indels and SSRs in the region between 9000 to 9600bp.

Authentication of tobacco cultivars is an important issue to be addressed to protect the interests of farmers as well as to provide quality

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product to consumers. Hence, major objective of this study was to discriminate and identify tobacco cultivars of commercial importance. In our study polymorphic regions of chloroplast genome were selected to amplify genes related to energy metabolism and photosynthetic machinery of plants [6]. We used chloroplast markers for the identification/authentication of 11 unknown samples of commercial tobacco cultivars. PCR amplification, sequencing, multiple alignment and phylogenetic reconstruction demonstrate that there exist at least 5 commercial varieties of tobacco that were mixed together. It also indicated evolutionary relationship among samples thus leading to the identification of tobacco samples.

2. MATERIALS AND METHODS

2.1. Plant Material Selection

Seeds of 13 commercial varieties were obtained from a local commercial tobacco company. These were designated as S1, S2, S3, S4, V1, V2, V3, V5, V6, V7, V8, V9 and V10. Plants were grown in the field of National Institute of Genomics and Advanced Biotechnology, National Agricultural Research Centre, Islamabad under suitable conditions. Plant materials (leaves) were harvested for DNA extraction.

2.2. DNA Extraction

Genomics DNA was extracted from 13 fresh tobacco leaf samples using CTAB method [7], and confirmed by running in gel electrophoresis. DNA samples were quantified using Nanodrop spectrophotometer (Thermo Scientific, Inc.) to check the quality as well as the quantity of extracted DNA.

2.3. Primer Designing

The chloroplast genome of tobacco was retrieved from NCBI database (https://www.ncbi.nlm.nih.gov/nucleotide/). The primers were designed manually by selecting the most polymorphic region in the chloroplast genome of tobacco. Primer sequences were forward primer 5'-GAGAAAGAGCTTCATTGCTTGGTGT-3' and reverse primer 5'-CCGGCTGGGTACTGACCAGACCAG-3'.

2.4. PCR Amplification

DNA samples were diluted and subjected to thermocycler (Applied Biosystems Inc) with primers for the amplification of the desired fragments. Each PCR reaction (50 µl) contained 10 ng DNA template, 5 µl 10 X reaction buffer, 5 µl MgCl2, 1 µl dNTPs, 1 µl of each primer, and 0.5 µl of Taq DNA polymerase (Promega, Madison, WI, USA). The PCR conditions set for amplification were 95°C for 5 min to activate taq polymerase, followed by 36 cycles of 94°C for 1 min (denaturation), 62°C for 1 min (annealing), 68°C for 1 min (extension) and a final 68°C for 5 min followed by 12°C. The PCR products were run on 1.5% agarose gel and confirmed.

2.5. Sequence Analysis, Multiple Alignment SNP Detection

After getting the results from PCR amplification and subsequent purification, 11 samples were sent to Macrogen (Korea) for sequencing. The sequence files obtained were edited and analyzed with Bio-Edit software [8]. Blastn was executed for target identification in NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were trimmed from both ends to remove any mismatched or flanking regions using the BioEdit software [8]. The resulting sequences were edited and analyzed with different tools. Sequence identity matrix was calculated. For this purpose the multiple alignment was loaded in ClustalX; output tree format option was chosen and % identity matrix was selected. Finally, a table was generated.

In order to observe the sequence conservation, multiple sequence alignment was performed using ClustalW program [9]. SNP (single nucleotide polymorphism) analysis of tobacco cultivars was carried out and a table was generated using BioEdit software [8].

2.6. Phylogenetic Analysis

Phylogenetic reconstruction was executed on the aligned sequences using Neighbor Joining method in MEGA 6.0 software [10] for finding the evolutionary connection among different samples. The evolutionary relationship, recombination, hybridization and homoplasmy are well exhibited

by phylogenetic network instead of a tree like illustration [10]. In this regard, a neighbor-net network algorithm was employed in SplitTree4 software [11] using default parameters.

3. RESULTS

3.1. Divergence in Amplified Sequences is Detectable among Different Tobacco Samples

In order to amplify DNA fragment, PCR was employed using standard conditions. Fig. 1 illustrates that it is possible to amplify a fragment of 650 bp from 11 out of 13 samples of tobacco. Two samples S3 and S4 could not be amplified mainly due to poor DNA concentration as leaves became already withered and dried. The band intensity or quantity and quality of amplified PCR products were high enough to be directly sequenced without purification step. Blastn search revealed that all the hits were targeting the tobacco chloroplast genome, thus indicating the authenticity of targets.

The target sequences were analyzed using Bio-Edit program. In order to detect the sequence conservation among different cultivars trimmed sequences were subjected to multiple alignments using Clustal W tool (Fig. 2). The alignments revealed conservation among all the sequences but different mutations including substitutions and even small deletions at the 5' and the 3' were also detected. But this may be due to selection of sequence length. Anyhow substitution mutations were most predominant. Table 1 shows that

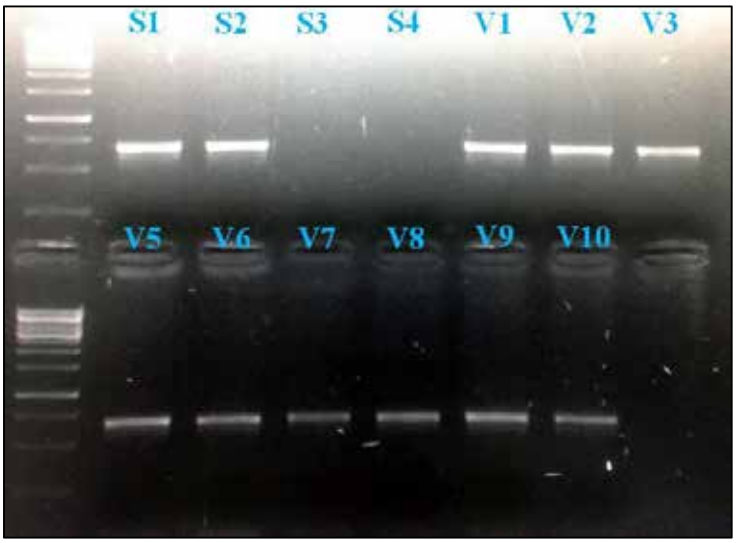

Fig. 1. Amplified PCR products have been labeled. 'M' denotes the 1kb ladder. S3 and S4 bands are missing because their DNA was degraded. Rest of the samples show clear bands

Table 1: Sequence identity matrix calculated using Bio-Edit software

Seq	S1	S2	V1	V2	V3	V5	V6	V7	V8	V9	V10	NCBI_ Z00044.2
S1	ID	98.8	90.4	98.3	98.3	98.6	99.3	99.0	99.5	99.0	99.1	98.1
S2	98.8	ID	90.9	99.1	98.5	98.5	99.5	99.5	99.0	99.1	99.0	98.0
V1	90.4	90.9	ID	91.0	90.4	91.0	90.7	90.7	89.9	90.0	89.9	89.5
V2	98.3	99.1	91.0	ID	99.0	97.6	99.0	98.6	98.1	98.3	98.1	98.5
V3	98.3	98.5	90.4	99.0	ID	97.6	98.3	98.3	98.5	98.6	98.5	98.8
V5	98.6	98.5	91.0	97.6	97.6	ID	98.6	98.6	98.5	98.3	98.5	97.5
V6	99.3	99.5	90.7	99.0	98.3	98.6	ID	99.6	99.1	99.3	99.1	98.1
V7	99.0	99.5	90.7	98.6	98.3	98.6	99.6	ID	99.1	99.3	99.1	98.1
V8	99.5	99.0	89.9	98.1	98.5	98.5	99.1	99.1	ID	99.5	99.6	98.6
V9	99.0	99.1	90.0	98.3	98.6	98.3	99.3	99.3	99.5	ID	99.5	98.5
V10	99.1	99.0	89.9	98.1	98.5	98.5	99.1	99.1	99.6	99.5	ID	99.0
NCBI_ 00044.2	98.1	98.0	89.5	98.5	98.8	97.5	98.1	98.1	98.6	98.5	99.0	ID

The percentage values of sequences showing maximum identity are highlighted in blue.

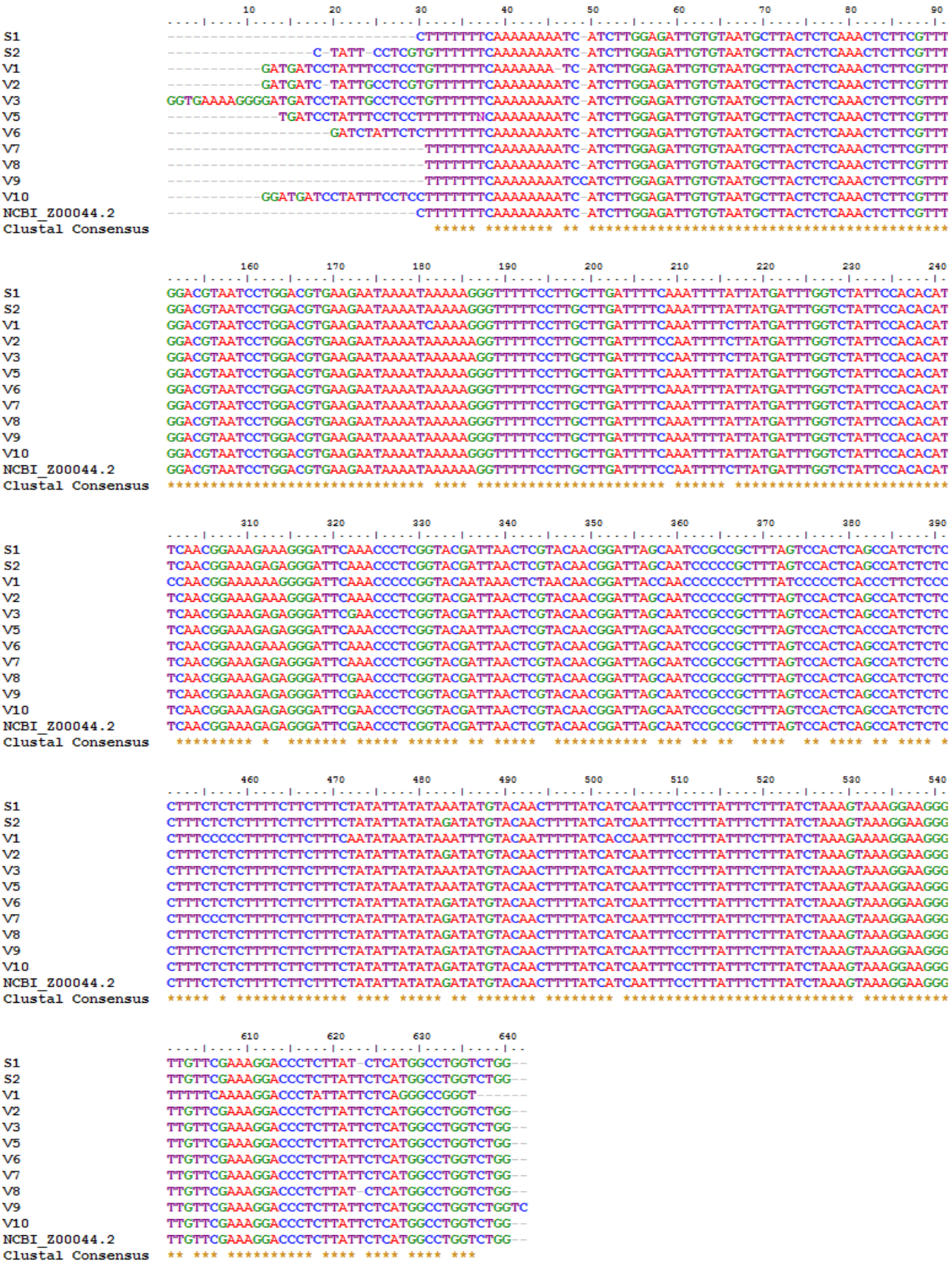

Fig. 2. Multiple alignment of tobacco samples with the reference sequence (NCBI_Z00044.2) using ClustalW program in BioEdit. Different colors represent four different nucleotides. Red is for adenine, blue for cytosine, green for guanine and purple for thymine. The consensus below indicates the conservation of nucleotides.

substitution mutations are distributed along the entire length of the selected region. A similarity matrix could better reveal substitution differences between the cultivars. Table 2 demonstrates that similarity ranged from 90.4 to 99.6 percent. A cut off value was set as indicator of different cultivar. Ninety nine percent identities indicate that these are the same cultivars but values below this index may be taken as different cultivars.

3.2. Chloroplast markers can differentiate tobacco cultivars

Phylogenetic analysis revealed the evolutionary relationship among the sequence samples thus leading to the identification of tobacco cultivars (Fig. 3). Our results indicate that some samples are closely related to each other; e.g., V8, V9, V10 and S1 are forming one clade showing that their origin is same, and hence are more close to each other than the rest of the samples. Similarly V6, S2 and V7 are forming second clade while V2, V3 and reference sequence (NCBI_Z00044.2) constitute the third clade (Fig. 3). The V1 and V5 are the outgroup sequences as they are present at the root of the tree indicating that they are the most divergent of all other samples. Comparison of V1 and V5 reveals that V1 is the most divergent of all the samples. The neighbor-net network analysis also demonstrated the similar results (Fig. 4), thus validating our results.

The above results allow us to infer that there exist at least 5 varieties of tobacco in these samples. The groups of samples in the same variety are listed in the table 3. V1 is the most distant cultivar. V5 is also separated from the rest of cultivars. But V2, V3 and NCBI sequence make further linage and can be considered as separate clade. A bigger cluster including V7, S2 and V6 is differentiated into distinct group. S1, V9, V10 and V8 constitute the 5th cluster that can be considered as a single variety. It can be extrapolated that these actually belong to the same variety as their sequences exhibit an evolutionary link i.e., having the same ancestral species. Hence there is chance of mixing, if these are considered as separate varieties physically. S2 and V7 may be originally treated as different but analysis indicated that this is the same variety but, names were given differently.

4. DISCUSSION

Molecular and bioinformatics data analysis of 11 unknown commercial samples of tobacco enabled us to ascertain that there exist at least 5 commercial varieties of tobacco that were mixed together. In this study it was possible to amplify a fragment of 650 bp through PCR. These sequences features mostly substitution mutations but deletions are also detectable at upstream and downstream terminal regions. Noman et al. [12] used similar CpDNA marker and found mutations in the variable regions of olive chloroplast. Marker sequence analysis of 965 unknown olive plants resulted in identification of 515 plants differentiated into 27 varieties successfully. Interestingly, the similarity matrix ranged from 90.4 to 99.6 percent. This indicates that differences not only account for existence of different varieties but may even different species. These observations were further validated through phylogenetic reconstruction.

Molecular phylogeny can be inferred in Muscari (*Asparagaceae*) species based on CpDNA sequences [13]. Liu et al. [14] successfully detected the intraspecific polymorphism in Phalaenopsis equestris cultivars. Their results are analogous to

Table 2: SNP (single nucleotide polymorphism) analysis of tobacco cultivars

	1	7	19	156	179	186	258	283	292	305	334	352	374	385	390	402	426	447	453	516	519	548	593
V1	G	T	-	G	A	C	A	A	A	A	C	C	A	C	A	A	C	A	A	A	A	A	T
V2	G	T	-	A	C	C	A	A	A	G	C	G	A	C	G	A	T	T	G	A	A	A	T
V3	G	T	-	A	C	C	-	G	G	G	G	G	A	C	G	G	T	T	A	A	A	A	T
V5	T	N	-	G	A	A	A	G	A	A	G	C	G	T	A	A	T	A	A	A	A	A	T
V6	T	T	-	G	A	A	A	A	A	G	G	G	A	T	G	A	T	T	G	A	A	A	T
V7	T	T	-	G	A	A	A	G	A	G	G	G	A	T	G	A	C	T	G	A	A	A	T
V8	T	T	-	G	A	A	A	G	G	G	G	G	G	T	G	G	T	T	G	A	A	A	-
V9	T	T	C	G	A	A	A	G	G	G	G	G	A	T	G	G	T	T	G	A	A	A	T
V10	T	T	-	G	A	A	A	G	G	G	G	G	G	T	G	G	T	T	G	A	G	A	T
S1	T	T	-	G	A	A	A	A	A	G	G	G	G	T	G	G	T	T	A	A	A	A	-
S2	G	T	-	G	A	A	A	G	A	G	C	G	A	T	G	A	T	T	G	A	A	A	T
NCBI	T	T	-	A	C	C	A	G	G	G	G	G	G	C	G	G	T	T	G	G	G	G	T

NCBI is a reference identifier. SNPs are highlighted in cyan.

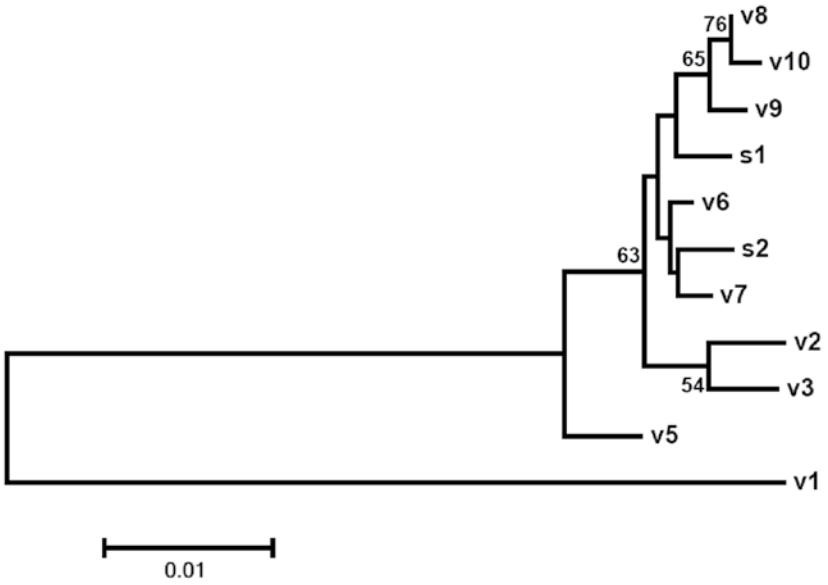

Fig. 3. Phylogenetic tree using Neighbor Joining algorithm (default parameters) to differentiate the samples. The bootstrap value was set to 1000 to check the robustness of tree. Scale bar shows nucleotides substitution per site.

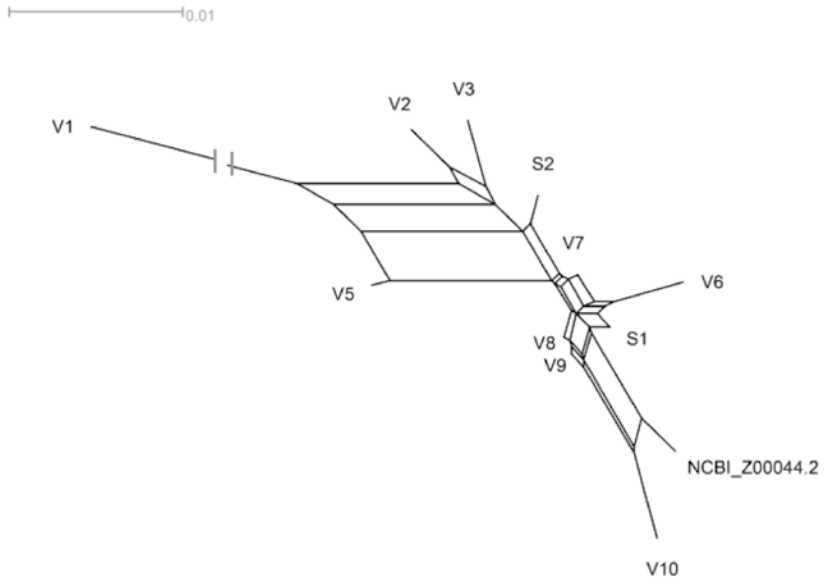

Fig. 4. Neighbor-net network analysis using SplitTree4 package (default parameters). Scale bar shows nucleotide substitution per site. The figure depicts V1 as outgroup OTU (Operational taxonomic unit) as compared to the rest of tobacco samples.

Table 3: Differentiation of tobacco samples

Variety No.	Identifiers included in the same variety
1	V1
2	V5
3	V2, V3
4	V7, S2, V6
5	S1, V9, V10, V8

A total of 5 varieties (cultivars) can be distinguished. V and S denote different samples.

our as they were able to evaluate 11 orchid cultivars that could be separated into six distinct groups. Our data demonstrates that there exist at least 5 varieties of tobacco in these samples. The groups of samples in the same variety are listed in the table 3. V1 is the most distant cultivar. V5 is also separated from the rest of cultivars. But V2, V3 and NCBI sequence make further linage and can be considered as separate clade. A bigger cluster including V7, S2 and V6 is differentiated into distinct group. S1, V9, V10 and V8 constitute the 5th cluster that can be considered as a single variety. It can be extrapolated that these actually belong to the same variety as their sequences exhibit an evolutionary link i.e., having the same ancestral species. Hence there is chance of mixing, if these are considered as separate varieties physically. S2 and V7 may be originally treated as different but analysis indicated that this is the same variety but, names were given differently. For further validation of these results morphological data is inevitable.

As high throughput sequencing is becoming cheaper and cheaper with time it is now possible to do the varietal profiling [15,16]. In this regard the role of SNP variations is inevitable. This is particularly useful for plant breeder's rights and varieties approval programs. Thus, chloroplast genome profiling is much cheaper and rapid strategies for varietal detection than nuclear genome that is still expensive and requires high quality expertise in genome informatics. In line with this assertion Zhang et al. [17] established the differentiation of rubber dandelion species from weedy relatives by using chloroplast high throughput NGS sequencing and molecular markers.

The results of this endeavour have repercussions in on-farm preservation of tobacco germplasm, and in availability of authentic plant material for the propagation of reliable varieties. This approach has implications in cultivar identification of any other species in plant kingdom.

5. CONCLUSIONS

Chloroplast markers used in this study have sufficient resolving power to discriminate 13 commercial samples of tobacco into 5 cultivars at intra-varietal.

6. ACKNOWLEDGEMENT

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Research Article

Spatial Variability in Proximate Composition of Lambsquaters (*Chenopodium album*) Grown in District Mianwali, Punjab, Pakistan

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Abstract: The current study was conducted to assess the proximate composition of *Chenopodium album* collected from different tehsils i.e. Mianwali, Esakhel, Piplan, of district Mianwali. The highest moisture (84.22±0.86%) and ash (29.96±2.12%) content was found in *Chenopodium album* collected from Piplan and Mianwali respectively. The maximum crude fiber (14.61±1.83%) and ether extract (27.11±0.21%) was reported in Piplan. The maximum crude protein (0.87±0.036%) and carbohydrates (11.67±1.38%) was observed in Esakhel while variation in proximate composition of *Chenopodium album* collected from different localities may be attributed to environmental factor (soil composition, water, temperature, light etc.) or may be due to spatial variation.

Keywords: Proximate composition, *Chenopodium album,* District Mianwali.

1. INTRODUCTION

This plant belongs to family Amaranthaceae which is used as vegetable [1], common names are as follows like Lambsquarters, Bathoo, Marathi, Fat-hen, Goosefoot, Papukurra. The height of plant is ranges from 10-120 cm. The leaves of *Chenopodium album* can be varied and are alternating in appearance [2].

Some important material such as alkaloids, nutrients, antioxidants, tannins, phenols, glycosides are present in *Chenopodium album* [3] which are very essential for the proper functioning of the body. This plant has retained aromatic compounds used in all over the world. Sugars, protein, fat, fiber are also present [4]. They are also important due to their effective efficiency of curing diseases with no side effects [5]. The Ethno-medicinal use of *Chenopodium album* plant is laxative, Anthelmintic, stimulant, and diuretic. The extracts of this plant are used to control digestive problems such as troubled stomach, constipation by enhancing the activity of the stomach [10].

It is stated that the district Mianwali, Punjab, Pakistan has great possibility of plant resources with wealthy heritage of native information about apply of these species. There is need of time to awake the local communities to guard and protect their plant resources by educating each other for sustainable growth and consumption.

The main aim of study is to explore the proximate composition of medicinal plant (*Chenopodium album*) in district Mianwali based on medicinal plant resources with particular importance on citations of native knowledge from neighboring communities.

2. MATERIALS AND METHODS

2.1 Area of study

Mianwali is situated between 32°-10° to33°-15° north latitude and 71°-08 to 71°-57° east. The most

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of these parts are the persistence of Potohar Pleatue and Salt Range the District Mianwali is surrounded on the north by Kheber Pukhtun Khawa and Attock district of Punjab, on the east by Kohat districts, the south by Bhakkar district of Punjab and on the west by Lakki, Karak and Dera Ismail Khan district of Kheber Pukhtun Khawa again. The climate of the Mianwali District as an entire is severe with extended hot summer and cold dry winters. Summer season from May to September and winter season from October till February. July is the hottest month in Mianwali with standard temperatures of 43°C while December and January are the coldest months with average minimum temperature 6°C -7°C [12].

2.2 Collection of samples

Plant leave samples were collected from three tehsils of district Mianwali i.e. Mianwali (T1), Esakhel (T2), Piplan (T3) for analysis. Each sample is comprised on three replicates, randomly picked up, wrapped in a particular black envelope and labeled.

2.3 Measurements

For proximate analysis plant leave samples were ground into fine powder. The AOAC method [6] is used to determine the proximate composition (moisture, dry matter, mineral matter, crude protein, crude fiber, ether extract, carbohydrates). Experiment was performed in Institute of Food Science and Nutrition, University of Sargodha, Sargodha.

2.3.1 Moisture: (%)

Fresh weight of plant samples. Then transferred these samples into the oven at 600°C for 24 hour. Then these samples were collected and weight noted on electrical balance.
The following formula was used for the determination of moisture contents

$$\text{Moisture \%} = \frac{\text{Dry weight of sample}}{\text{Fresh weight of sample}} \times 100$$

2.3.2 Mineral matter: (%)

Dehydrated samples were scorched with the oxidizing blaze until fumes formed. The plant samples were then ignited at 550°C furnaces to flame all the whole matter.

The given formula was used to determine the mineral matter percentage composition

$$\text{Mineral matter} = \frac{\text{weight of ash}}{\text{weight of sample}} \times 100$$

2.3.3 Ether extract: (%)

Soxhlet apparatus was used for extraction of crude fat with petroleum ether (45°C – 60°C). This method was used to eliminate the part that was ether soluble. Plant leave samples were used in this process. It was dehydrated up at 70°C until the stable weight achieved.

The following formula was used for the determination of ether extract contents

$$\text{Ether extract} = \frac{\text{Wt of flask with ether extract} - \text{Wt of empty flask}}{\text{weight of sample}} \times 100$$

2.3.4 Crude fiber: (%)

Acid-base digestion method was used to determine the crude fiber composition. Firstly, the samples were dried into oven. Soxhlet apparatus was used to remove the fat and digested the plant samples in 1.26% NaOH and H_2SO_4 separately to remove acid-base contents from the samples. Then the samples were washed three to four times by means of distilled water, transferred in Petri dishes and placed in kiln on 105°C for one day. The variance among the weights of the plant samples were the contents of crude fiber.

The following formula was used for the determination of crude fiber content

$$\text{Crud fiber \%} = \frac{\text{Wt of flask with sample after drying} - \text{Wt of empty flask}}{\text{weight of sample}} \times 100$$

2.3.5 Crude protein: (%)

The Kjeldhal method was used for determination of protein, first total nitrogen was determined by using

Kjeldhal apparatus [7], and then protein contents were obtained by multiplying nitrogen to a factor of 6.25.

Crude protein = % Nitrogen × 6.25

2.3.6 Carbohydrates: (%)

Carbohydrates concentration were determined by subtracting ether extract, mineral matter, protein, fiber and moisture contents from 100.

Carbohydrates = 100 - (Moisture, mineral matter, crude protein + ether extract + crude fiber).

2.4 Statistical analysis

Descriptive statistics was carried out using Microsoft Excel 2007 [13].

3. RESULTS

The results showed that there is significant variations in proximate composition of *Chenopodium album* are present. However, the moisture and crude fiber content in *Chenopodium album* varies from 84.22±0.86, 14.61±1.83 (T3) to 80.04±2.32, 11.72±0.43 (T1) with a mean value of 82.53±1.23, 12.98±1.12 respectively, while the mineral matter and ether extract varies from 29.96±2.12 (T1), 27.11±0.21 (T3) to 29.12±1.92 (T2), 11.72±0.43 (T1) with a mean value of 29.64±1.76, 24.98±0.57 respectively. Maximum crude protein content was found in T2 (0.87±0.036) while the lowest in T1 (0.18±0.006) with a mean value of 0.42±0.02, while carbohydrates content was found to be highest in T2 (11.67±1.38) and minimum was noted in T1 (6.94±0.46) with a mean value of 9.30±0.64.

4. DISCUSSION

The human body is basically water. Babies are comprised of 70 percent water, while grown-up guys are 60 percent and females are 55 percent [14]. Water gives the medium to make your blood, helps move nourishment through your stomach related tract and expels squander from each cell in your body [15]. Water hydrates the body by filling in as an oil to soak joints, and furthermore ensures your eyes, mind and spinal line [16]. The stomach related framework utilizes water for indispensable liquids, for example, blood, salivation and stomach related liquids to help in the transportation of supplements and evacuation of waste items. Water helps move your sustenance through your digestive organs, which is vital in avoiding constipation [17]. Results regarding moisture content were in collaboration with the findings of Hussain et al [18] and Singh et al [19]. Moisture content in different samples vary from 84.33±0.01 to 87.5±1.1 in *Chenopodium album*. The mature plant of *Chenopodium album* was retained 97.4 ± 0.1% moisture content [8]. While according to Gqaza et al [9] that *Chenopodium album* was retained 9.13% moisture content in South Waziristan area of Pakistan.

Chenopodium album contain many important minerals that are very essential for proper functioning of the body [20]. They are important for our survival as part of hemoglobin, myoglobin., acting as a catalyst in many biological reactions [21], are also important for our nervous system (Transmission of messages from one part of body to another), also important for proper food digestion [22], metabolism, utilization of all nutrients in food [20]. Results regarding mineral matter content are in collaboration with the findings of Singh et al [19] and Hussain et al [18]. Mineral matter concentration in different samples of *Chenopodium album* vary in range from 2.07±0.3 to 22.15±0.09 in fresh and dehydrated leaves, while 23.25±0.25% also noted by Adedapo et al [23]. In Okra, Amaranth vary from 9.036±0.01% to 22.84±0.04% respectively [9].

Table 1: Proximate Composition of Bathoo (*Chenopodium album*) from three different tehsils of Mianwali District

Tehsils	Moisture	Mineral matter	Crude fiber	Ether extract	Crude protein	Carbohydrates
T1	80.04±2.32	29.96±2.12	11.72±0.43	21.21±0.61	0.23±0.029	6.94±0.46
T2	83.34±0.63	29.12±1.92	12.63±1.11	26.64±0.89	0.87±0.036	11.67±1.38
T3	84.22±0.86	29.84±1.31	14.61±1.83	27.11±0.21	0.18±0.006	9.31±0.09
Mean	82.53±1.23	29.64±1.76	12.98±1.12	24.98±0.57	0.42±0.02	9.30±0.64

Dietary fiber is that piece of plant material in the eating regimen which is impervious to enzymatic absorption which incorporates non-cellulosic and cellulosic polysaccharides, for example, pectic substances, mucilage, hemicellulose, gums and a non-starch segment lignin. The eating regimens wealthy in fiber positively affect wellbeing in relieving a few problems. It is also useful for stomach related framework, enhances inside working and soothes bowl drive in patients [24], may likewise go about as a defensive factor in malignancy, decreasing the measure of cancer-causing agent that interacts with the gut divider, good for diabetic patients [27, 28], reduce the risk of heart failure by reducing the cholesterol level [29], lowering both serum cholesterol and triglycerides [24]. Results regarding crude fiber were in collaboration with the findings of Dai et al [11] and Singh et al [19]. crude fiber content in *Chenopodium album* noted as 13.92% in Nigeria. It varies from 0.81±0.08 to 6.26±0.11 in fresh and dehydrated leaves of *Chenopodium album*. In Okra, Ribbed loofah, Amaranth vary as 14.71±0.02, 10.25±0.01, 10.13±0.05 respectively.

Ether extract or fats (crude) are a kind of supplement that you get from your eating regimen. It is essential to eat a couple of fats, anyway it is in like manner dangerous to eat excessively. These makes body energetic that it needs to work truly. These are second source of energy for body [17]. Fat in like manner need to keep skin and hair strong, moreover holds fat-dissolvable (vitamins K, D, A and E), furthermore fills your fat cells and ensures body to help keep you warm. The fats your body gets from your sustenance give your body crucial unsaturated fats called linoleic and linolenic destructive. They are called "essential" in light of the fact that your body can't make them itself, or work without them [30]. Your body needs them for psychological well-being, controlling disturbance, and blood coagulating [21].

Results regarding crude fat were in collaboration with the findings of Adedapo et al [23] and Singh et al [19]. The crude fat content in *Chenopodium album* varies from 0.63 to 13.92%. In *Bidens pilosa* noted as 6.0± 1.0.

Results regarding crude fat were in collaboration with the findings of Adedapo et al [23] and Singh et al [19]. The crude fat content in *Chenopodium album* varies from 0.63 to 13.92%. In *Bidens pilosa* noted as 6.0± 1.0.

Protein is key to good health - it's involved in transport of oxygen, maintains the health of muscle tissues, improve immune functions. It also important for growth and development in children, teens, and pregnant women. They are large, complex molecules that have many critical roles in the body. They are composed of amino acids and bound together by peptide bonds. They also play important role in the formation of DNA, enzymes, neurotransmitters, antibodies, hairs and nails [30]. Results regarding crude protein were in collaboration with the findings of Singh el al [19] and Gqaza et al [8]. The crude protein in different samples of *Chenopodium album* rang vary from 3.7±0.12 to 32.95±0.24. In *A. viridus, L. acutangula* noted as 16.41±0.03, 13.43±0.06 respectively reported by Hussain et al [18].

Much the same as your auto needs fuel to influence it to run, your body needs fuel to influence it to go. your body doesn't keep running on gas - it keeps running on sugars. Starches which are present in our diet like grains, milk products, beans, vegetables and fruits, are by a wide margin your body's most loved wellspring of vitality. The primary capacity of starches is to provide energy for the brain and body [31]. A sufficient admission of carbs likewise saves proteins and assists with fat digestion. The greater part of your body cells utilizes the straightforward sugar glucose for vitality, yet your mind is especially needing glucose as a vitality source. Along these lines, we can include that a critical capacity of carbs is providing vitality to the cerebrum. On the off chance that you have ever gone on a low-carb eating routine and felt like your cerebrum was foggy for a couple of days, at that point you encountered exactly how imperative starches are to appropriate mind work [32]. Another capacity of sugars is to keep the breakdown of proteins for vitality. By expending adequate measures of starches in your eating routine, you guarantee that your body can meet its vitality needs, yet in the event that your admission of carbs is too low or you are utilizing them up too rapidly, for example, during forceful work or exceptional exercise, at that point your body is compelled to break down proteins for vitality [33]. Results regarding carbohydrate content are in collaboration with the findings of Gqaza et al

[9] and Singh et al [19]. Carbohydrate content in different samples of Chenopodium album vary from 4.0±0.0 to 34.46±0.85 %. Some others also noted in *Chenopodium album* range from 29.41±0.1 to 41.58±0.3 %.

5. CONCLUSION

The results of the analysis suggest that observed plant collected from all tehsils contain good source of nutritional composition (proximate) and may use as useful forage for animals but there is fluctuation in nutritional content (proximate) of *Chenopodium album* present in all tehsils which may attributed to different environmental factors (Soil composition, Water, Temperature, Light) or may be due to spatial variation. Exploration of nutritive value of this plant will not only be of economic importance but would be a step toward better utilization of these plants as phytomining, biofuel production or may be used for additional feed and medicine production (homeopathic, allelopathic).

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Research Article

Effect of Dietary Supplementation with Propylene Glycol on Blood Metabolites and Hormones of Nili-Ravi Buffalo Heifers

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Abstract: The current study was designed to investigate the effects of propylene glycol (PG) on blood metabolites, feed intake and fertility in buffalo heifers. For this purpose, 12 Nili-Ravi buffalo heifers were selected and divided into three groups A, B and C. Group A was kept as control while group B was supplemented with 150 g of PG and group C was supplemented with 300 g propylene glycol per animal per day for 30 days. Blood samples were taken on day 0, then 1st, 3rd, 5th and 7th week after initiation of PG supplementation. The serum glucose, triglycerides, total protein, albumin, alanine transaminase, total cholesterol, blood urea nitrogen, oestrogen and progesterone were determined. Results revealed that serum glucose level significantly ($P<0.05$) increased in both treated group as compared to control group. Total protein, alanine aminotransferase (ALT), albumin and progesterone concentrations increased significantly ($P<0.05$) in treated groups as compared to control group. In treated groups serum cholesterol, blood urea nitrogen, and triglycerides are significantly decreased ($P<0.05$). Based on results of the present and previous studies, it was concluded that feed supplementation of propylene glycol on blood metabolites and reproductive hormones showed remarkable changes.

Keywords: Supplementation, Propylene Glycol, Blood metabolites, Nili-Ravi Buffalo

1. INTRODUCTION

Buffalo is found in more than 50 countries of the world. It has capacity to adopt itself in changing environment, atmosphere, geography and tropical conditions. In Pakistan, Buffalo mainly raised for milk production, sharing 65% of total milk production and their male calves contribute in meat industry as well [1]. Buffalo milk is preferred in whole country and sells at a high rate as compared to cow's milk because of higher milk fat and solid constituents. The average milk yield per lactation of Nili-Ravi buffalo is 2430 liters but it has capacity to yield above 5000 liters per lactation [2]. Buffalo is an important element of livestock sector of Pakistan as it provides livelihood to a considerable portion of people living in villages. Among major economic problem responsible for poor reproductive performance of buffalo, late puberty, long calving intervals, and poor estrus expression - especially during summer months and seasonality are important

Body metabolic and nutritional status of animals are associated with reproductive efficiency, nevertheless, its mechanism is unknown [3]. Concentration of blood metabolites, such as leptin, growth hormone, insulin-like growth factor (IGF-1) and insulin regulate the central nervous system to control the secretion of gonadotrophin. Blood insulin level and ovarian activity of dairy cows in postpartum period can be regulated by varying dietary starch and fat supply [4]. Plasma level of insulin and glucose are increased after dietary propylene glycol supplementation [5]. After oral supplementation a minor amount of propylene glycol is metabolized to propionate. Most of propylene glycol (PG) by pass the rumen

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unchanged to be converted to glucose in the liver. PG is converted to glucose in liver via lactaldehyde pathway and later oxidation to lactate take place. Propionate in liver is converted to pyruvate and in glucose via oxaloacetate. Propylene glycol increases the plasma concentration of glucose and insulin but its underlying mechanism is not understood [6]. Propylene glycol administration elevates IGF-1. IGF-1 plays very important role in steroid hormone synthesis, maturation of follicle, ovulation, fertilization, attaching of concept us to uterine wall and in development of embryo [7]. An elevated concentration of insulin during the oestrous cycle can increase the number of follicles [8]. Animals supplemented with propylene glycol exhibited their first ovulation after parturition as compared to control group. Propylene glycol supplemented in feed or administered orally improves fertility and metabolic status of dairy animals [9]. Keeping in view the limited information about the propylene glycol on health of buffalo under the climatic conditions of Pakistan, this study was planned.

2. MATERIALS AND METHODS

2.1 Experimental Animals

The study was carried out to determine the effects of dietary supplementation of propylene glycol on blood metabolites and reproductive organs of buffalo heifers having age from 24-30 months. For this purpose, 12 healthy buffalo heifers were randomly allocated into 3 groups, the experiment was carried out at Proka Farm University of Agriculture Faisalabad. Group A was kept as non-treated while B and C group were supplemented with 150g and 300g of propylene glycol, respectively, mixed in routine ration, which consisted on wheat straw, berseem and concentrate mixture for a period of 30 days. Propylene glycol is a colorless fluid and sprayed over ration.

2.2 Blood collection

Animals were restrained in cattle crush at farm for the collection of blood samples. Blood samples (10ml) were collected from jugular vein, under hygienic precautions, into gel and clot activator and serum was stored at 4 °C for further use [10]. Blood collection was done on day 0, after 1st, 3rd, 5th and 7th week of PG supplementation.

2.3 Biochemical and hormonal Analysis

Analysis was carried out to determine various biochemical compounds i.e., glucose, cholesterol, total proteins, albumin, ALT, triglycerides and blood urea nitrogen in the serum using commercially available standard kits and hormonal profile (estrogen and progesterone) by using Enzyme Linked Immunosorbent Assay (ELISA) Germany.

2.4 Statistical Analysis

Mean values (± SEM) of concentration of different parameters observed were calculated. Data were analysed by analysis of variance using general linear model procedure [11]. Duncan's Multiple Range Test [12] was applied for multiple mean comparison where ever significance variation indicated by ANOVA.

3. RESULTS

The results of the study showed that serum glucose levels were higher ($P<0.05$) in treatment groups B (51.49 ± 9.60) and C (52.54 ± 9.57) as compared to control group (39.42 ± 0.76).The group B (8.022 ± 0.17 g/dl) and C (8.310 ± 0.17 g/dl) had significantly higher total serum protein concentration than group A (6.920 ± 0.05 g/dl). The group B & C were (3.140 ± 0.09, 3.259 ± 0.07), significantly increased ($P<0.05$) as compared to A (3.14 ± 0.09). serum triglycerides were significantly decreased ($P<0.05$) both in groups B & C (43.567 ± 4.53&41.281 ± 6.47) treated with PG as contrast to mean value of control group A (48.637 ± 0.40). Serum cholesterol levels were significantly lower in both groups as compared to control group A (51.851 ± 1.14), while between group B & C (40.164 ± 6.48 & 39.591 ± 6.74) these were non-significant with each other. Blood urea nitrogen were decreased both in treated group as compared to control group. Level of serum ALT concentration was increased in groups treated with 150 gm & 300 gm of PG as compared to control group. Serum progesterone level significantly increased ($P<0.05$) in groups B & C (5.301 ± 3.78 & 5.415 ± 3.55) as contrast to control group A (1.478 ± 0.17). Serum estrogen level increased with PG treated groups (6.229 ± 2.73 & 6.5552 ± 2.80) as compared to control group A (3.457 ± 1.41).

Table 1: Serum biochemical metabolites and hormonal profile in control and treated groups of buffalo heifers treated with propylene glycol.

Serum parameters	Group A	Group B (150g PG)	Group C (300 g PG)
Serum glucose (mg/dl)	39.44 ± 0.76^{C}	51.49 ± 9.60^{B}	52.54 ± 9.57^{A}
Serum total protein (g/dl)	6.90 ± 0.09^{B}	8.07 ± 0.69^{A}	8.20 ± 0.74^{A}
Serum albumin (g/dl)	3.14 ± 0.09^{B}	3.25 ± 0.07^{AB}	3.45 ± 0.10^{A}
Serum triglycerides (mg/dl)	48.63 ± 0.40^{A}	43.56 ± 4.53^{B}	41.28 ± 6.47^{C}
Serum cholesterol (mg/dl)	51.85 ± 1.14^{A}	40.16 ± 6.48^{B}	39.59 ± 6.74^{B}
Blood urea nitrogen (mg/dl)	42.22 ± 1.45^{A}	36.77 ± 4.39^{B}	35.59 ± 5.53^{C}
Serum ALT (U/L)	30.74 ± 0.63^{C}	36.62 ± 4.54^{B}	37.76 ± 6.95^{A}
Serum progesterone (ng/ml)	1.47 ± 0.17^{B}	5.30 ± 3.78^{A}	5.41 ± 3.55^{A}
Serum estrogen (pg/ml)	3.45 ± 1.41^{B}	6.22 ± 2.73^{A}	6.55 ± 2.80^{A}

4. DISCUSSION

In this study, the authors attempted to clarify the potential effects of propylene glycol on serum biochemical metabolites in heifer buffalo. Serum glucose concentration was significantly ($P<0.05$) raised by supplementation of 150 and 300 gm of propylene glycol per animal per day in buffalo heifers. Our results of glucose concentration are in line with Gamarra et al. [13], who reported that concentrations of glucose were raised at 4 h after PG supplementation for the groups administered with propylene glycol 150 and propylene glycol 300 g as compared with day 0. Kristensen et al. [14] suggested that propanol may setup an insulin resistance thus hinder the uptake of glucose by insulin-sensitive tissues and thereby cause glucose concentration to increase. The results of present study showed significant increase ($P < 0.05$) in total serum protein concentration in treatment groups, which in agreement with Ayoub et al. ([15] those who reported that total protein is significantly increased in cows supplied 200 ml propylene glycol 30 days postpartum [16]. Increase in total protein concentration is the result of dietary feeding or ruminal utilization of protein constituents in the feed [17]. Serum albumin and ALT concentration in groups treated with PG were significantly increased ($P < 0.05$). Serum albumin is early nutritional marker of protein level [18]. Ayoub et al. [15] reported that propylene glycol administration had no effect on albumin concentration while supplied 100ml and 200 ml propylene glycol to healthy pregnant cows from 30 days before calving to 30 days of lactation. The primary (principal) indicators of liver lesion and disorder in the liver function are revealed by its enzymes aspartate aminotransferase (AST), bilirubin, ALT and blood metabolites [19]. They also reported that propylene glycol has ability to reduce the liver enzymes concentration after its supplementation.

Significantly reduced level of triglycerides in treated groups ($P < 0.05$) are in line with the results of Ayoub et al. [15]. They also found that triglycerides concentration was significantly reduced in animals supplied with 200 ml of propylene glycol at 30 days postpartum. Results of our study are in line with Chiofalo et al. [20]. Nazifi et al. [21] who also studied decrease in triglyceride concentration. In dairy animals the variation in triglycerides concentrations are principal variable for fatty liver, because triglycerides generation and storage is the major metabolic fate of fatty acids when the oxidation capacity of liver is increased [22].Serum cholesterol concentration decreased significantly in buffalo heifers in group B and C supplied with 150 g and 300 g propylene glycol per day per animal respectively, which in agreement with the results of Ayoub et al. [15] they found that propylene glycol administration reduced cholesterol concentration after parturition. Grummer and Carroll [23] described the importance of cholesterol as a precursor of ovarian

steroidogenesis. Schlumbom et al. [24] suggested that propylene glycol administration has capacity to elevate the cholesterol concentration after the end of supplementation due to the reduced response of target tissues towards insulin that together with raised mobilization of fatty acids from adipose tissue, which make available new sources for fetal growth. Significant reduction ($P<0.05$) was observed in urea nitrogen level which revealed by results that propylene glycol has an encouraging effect on negative energy balance, [25]. Findings of our study support the results of Ayoub et al. [15], Rukkwamsuk [26], Lien et al. [10], Hidalgo et al. [27], Duncan [12] who found that propylene glycol supplementation decrease blood urea nitrogen in treated animals. Serum urea nitrogen is the sign of crude protein intake [28] as well as energy and protein balance in ruminant's diet. During the present study while measuring the blood reproductive hormones it was noticed that serum progesterone concentration raised significantly which are in line with Berlinguer et al. [29].

A supportive effect of a glycogenic supplement on progesterone concentrations was present in sheep. Gamarra et al. [13] also stated that short term propylene glycol supplementation mixed in feed affects concentration of metabolic hormones, progesterone concentration and the number of small follicles. Raised progesterone can be procured from metabolism in the liver and other organs and possibly there may be release of progesterone from the adrenal gland but the basic source of progesterone in blood from non-pregnant buffaloes is considered to be the Corpus Luteum [30]. Estrogen concentration increased in treated groups compared to control group but variation was insignificant but estrogen increased in animals that exhibited cyclicity during the trial that's why estrogen increased in treatment groups. Our results are comparable with results of Gamarra et al. [13] Those found that there was no effect of propylene glycol administration on oestradiol concentration.

5. CONCLUSION

It was concluded that feed supplementation of propylene glycol to buffalo heifers having age 24-30 months improved and estrogen. It may be proposed that propylene glycol may be used for welfare of health of buffaloes.

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Research Article

Utilization of Wheat (*Triticum aestivum*) and Berseem (*Trifolium alexandrinum*) Dry Biomass for Heavy Metals Biosorption

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Abstract: Sorption capacity of wheat (*Triticum aestivum*) and berseem (*Trifolium alexandrinum*) biomass was checked against heavy metals i.e. copper (Cu), cadmium (Cd), lead (Pb) and chromium (Cr). Dry biomass was introduced in Erlenmeyer flasks of 250 ml volume to study the effect of varying initial metal concentration, pH and contact time. The filtrates were analyzed using flame atomic absorption spectrophotometer. The adsorption data of wheat and berseem biomass was fitted to Langmuir and Freundlich models. Results exhibited the maximum initial metal concentration (200 mg/L), pH 5 and 9, contact time 60 and 120 min are suitable for biosorption using wheat and berseem plants biomass. It can be recommended from the present study that berseem and wheat biomass can be used for waste water treatment in a cost effective and easy mode.

Keywords: Sorption, Dry biomass, Environment, Green Chemistry, Phytoremediation, Adsorption models

1. INTRODUCTION

Animals and plants have been affected by heavy metal contamination on a global scale. The constant threat imposed by heavy metals on human and animal health can be minimized by utilization of the different crops harvested in the soils affected by heavy metals. Such crops, either food or fodder possess an inherent ability for accumulation of heavy metals by different mechanisms [1, 2]. The potential of fodder crops accumulating heavy metals is influenced by number of factors e.g. pedospheric composition, climatic factors, types of agricultural chemicals used, quality of the irrigation water, the type of plant utilized and the parts of the plants used [3]. Regions located in close proximity to rivers are particularly at risk of pollution due to heavy metals. River water affected by heavy metals when used for irrigation of crops can also influence the soils negatively. Different heavy metals are the essential constituents of different metabolic pathways of plants and animals. Nevertheless, the amount of heavy metals beyond threshold can be lethal and associated with the disruption of the organisms normal physiological functioning [4,5]. Particularly, they can induce extreme toxicity and carcinogenicity on different living organisms [6].

Heavy metal contamination of pedospheric compartment is an ever growing issue and can be attributed to a myriad of anthropogenic activities e.g. agrochemicals, waste dumping etc. [7, 8]. Soil based heavy metal contamination is highly a serious concern since the heavy metals can be translocated into the plants from soils and later transferred to the higher trophic levels of food chain. The translocated heavy metals can be removed and used as a strategy for heavy metal remediation. Such an approach is referred as phytoremediation [9, 10]. The use of energy crops for phytoremediation is an eco-friendly approach marked by plants being inexhaustible energy resource. The integrity of ecosystems can be managed and enhanced by phytoremediation as an effective strategy [11]. Phytoremediation plants are carbon sinks [12] that not only cleans the environment but also reduces the heavier costs associated with other physicochemical modes of remediation. The process of phytoremediation can be further enhanced by utilization of alternating current (AC) [13]. Researches have also been

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done by inoculating phytoremediation plants e.g. sunflower with different fungal species for enhancing its heavy metal uptake potential [14, 15].

Industrial wastewater is a source of heavy metals which is responsible for water and soil pollution [16]. The use of this polluted water creates adverse impact on all the living organism and environment and ultimately metal ions can enter into the human food chain from the soil or water, disturb the bio-chemical processes and finally lead to serious effects on living organisms [17]. Heavy metals are possible to remove from aqueous solution by physical, chemical and biological technologies but conventional methods for removal of metal ions from aqueous solution have been suggested, such as chemical precipitation, filtration, ion exchange, electrochemical treatment, membrane technologies, adsorption on activated carbon and evaporation etc. Biosorption is a physico-chemical process and includes absorption, adsorption, ion exchange, surface complexation and precipitation [18]. For the elimination of organics and metals at large scale a huge number of materials have been studied for their capability to be used as biosorbent. On the whole, the experienced biosorbents are categorized as microbial [19] e.g. bacteria, fungi, yeast, algae [20], industrial wastes [21], agricultural wastes [22] and other polysaccharide materials. For this study dry biomass of wheat and berseem were collected from farmer fields. The aim of study was to determine the biosorption capacity of wheat and berseem dry biomass and serve as better biosorbent for environment reclamation.

2. MATERIALS AND METHODS

2.1. Plants sample collection and preparation of biosorbent

Wheat and berseem samples were collected from farmers' fields. After collection wheat and berseem plant samples were washed with tap water and distilled water. Washed samples were air dried and cut into small pieces with the help of knife. Now the plant samples were oven dried by keeping them in oven at 65.8 ℃ until constant weight. Dried plant samples were grinded with the help of grinding machine. Ground samples were prepared as biosorbents and were ready to use for biosorption experiments. The biosorbents prepared from wheat and berseem plants were packed in air tight polythene bags and then put into desiccators for use in biosorption experiments.

2.2. Metal biosorption experiment

Biosorption of wheat and berseen dry biomass was checked at different pH (5, 7 and 9), different metals (Cu, Cr, Pb, Cd) concentrations (50 ppm, 100 ppm and 150 ppm and 200 ppm) and different time contact (30, 60, 90 and 120 minutes). Metal stock solutions Cu, Cr, Pb and Cd were prepared in 250ml conical flask. In conical flask 0.25 gm of plant dry biomass was added individually in 60 ml of Cu (II) solutions at different concentrations (50 ppm, 100 ppm, 150 ppm and 200 ppm). The flasks were covered with aluminium foil and were agitated on a rotator shaker for varying time intervals (30, 60, 90, 120 minutes) at 154 rounds per minute, at room temperature and at pH value 7. Similar experiment was conducted with different pH values (5, 7 and 9) while keeping the metal concentration constant at 100 ppm. After each experiment, the mixture was filtered through Whattman filter paper No.1 and the filtrate was analysed for metal concentration by flame atomic absorption spectrophotometer. In order to run the samples in atomic absorption spectrophotometer, the concentration of the metal solution was reduced by doing metal solution dilution after each experiment. At the end of each experiment, triplicates of samples were taken. The samples were stored in plastic bottles and were analysed further in atomic absorption spectrophotometer. Similar procedure was repeated for Cr (III), Pb (II) and Cd (II). Berseem and wheat biosorbents were experimented separately but procedure followed was the same for both biosorbents. The heavy metal concentrations in the filtrate were analysed by flame atomic absorption spectrophotometer. Biosorption capacity i.e. the amount of metal ions (mg) sorbed by plant biomass (g) was calculated by using the equation 1.

$$\mathbf{Q} = \frac{(\mathbf{C_i} - \mathbf{C_f})}{\mathbf{mV}} \qquad (1)$$

Where Q = metal ion bioadsorbed (mg g-1) of biomass, C_i = initial metal ion concentration (mg l^{-1}), C_f = final metal ion concentration (mg l^{-1}), m = mass of plant biomass in the reaction mixture (gm) and V = volume of the reaction mixture (l). Statistical analysis of data was done on Microsoft Excel (2013) by using Langmuir isotherm (1916) and Freundlich equation (1909).

3. RESULTS AND DISCUSSION

3.1. Biosorption capacity of Wheat and Berseem biomass at initial metals concentration

For metal removal, agricultural waste supplies are an outstanding source being cellulosic in nature. These have diverse functional groups (hydroxyl, phenolic, carboxyl, amino and, acetamido) and form chelates and metal complexes because of having affinity for metal ions. Lignin and cellulose are the major constituents of agricultural waste material with other polar functional groups (aldehydes, alcohols, carboxylic acid, ketones and ethers) and support metal complexation resulting in uptake of metals. Rice fibre saw dust, wool, orange peel, soya bean and cotton hulls, banana pith, pine bark, wood and peat have been verified to uptake heavy metals from the wastewater. The agro based waste biosorbents are economical, non-risky and vast resources, which are particularly selective for heavy metals and can be simply disposed by burning [23].

3.1.1. Copper (Cu)

Wheat biomass showed a gradual increase in biosorption tendency with increasing initial copper concentration. Maximum biosorption by wheat biomass was noticed 151.4 mg/g (76%) and for berseem biomass 48.03 mg/g (74%) at initial copper concentration of 200 mg/L. The comparison expressed that wheat biomass behaved as better biosorbent as compared to berseem biomass (Figure 1).

3.1.2. Cadmium (Cd)

The wheat biomass showed a gradual increase in biosorption tendency with increasing initial cadmium concentration. Maximum biosorption by wheat biomass was noticed 146.2 mg/g at initial cadmium concentration (200 mg/L). In case of berseem biomass, maximum biosorption (153.8 mg/g) was shown at initial cadmium concentration. This comparison expressed that berseem biomass behaved as a better biosorbent as compared to wheat biomass (Figure 1).

3.1.3. Lead (Pb)

Maximum biosorption by wheat biomass was noticed 164.8 mg/g at initial Pb concentration of 200 mg/L. In case of berseem biomass, optimum Pb removal (62.5%) was observed at 200 mg/L of initial Pb concentration. Comparison of wheat and berseem biomass as biosorbents was done in terms of biosorption capacity when initial Pb concentration was conditioned. This comparison expressed that wheat biomass behaved as better biosorbent as compared to berseem biomass.

3.1.4. Chromium (Cr)

Maximum biosorption by wheat biomass was noticed 177.0 mg/g at initial chromium concentration of 200 mg/L. So, best Cr removal (88.5%) was observed at 200 mg/L (initial Cr concentration). In case of berseem biomass, maximum biosorption (142.34 mg/g) was also shown at initial chromium concentration of 200 mg/L. So, in case of berseem biomass, optimum Cr removal (71%) was observed at 200 mg/L of initial Cr concentration. This comparison expressed that wheat biomass behaved as better biosorbent as compared to berseem biomass. In the present research, biosorption is dependent on initial metal concentration. According to this investigation, there was increase in metal uptake with increase of initial metal concentration. Sorption capacity of wheat and grass biomass gradual increase in Cr (III) removal with increase in the initial concentration of Cr (III).

Biosorption potential of banana peel against Cu, Pb, Zn and Ni increased with initial metal concentration [24]. Biosorption potential of spinach stalk and pawpaw seed against Mn and Pb ions metal uptake increases as the initial metal ion concentration increases. Biosorption potential of agro based waste material maize tassel, for removal of Cd (II) and Cr (VI) increased metal uptake with increase of initial metal concentration up to 300 mg/L. Sorption in this case was also found dependent on increasing initial metal concentration [25].

3.2. Effect of contact time for biosorption capacity of Wheat and Berseem biomass

3.2.1. Cu

Wheat biomass showed different biosorption potential at different time intervals. Initially, at 30 minutes contact time, wheat biomass removed 66% (65.91 mg/g) Cu ions. While at 60 minutes, 77%

(76.77 mg/g) Cu biosorption was being observed. But at 90 minutes time interval, only 62% (62.14 mg/g) Cu removal was shown. However, at 120 minutes, 69% (68.59 mg/g) uptake of Cu was noticed. On the basis of above results, it is obvious that the optimum time for Cu biosorption by the wheat biomass is 60 minutes. In case of berseem biomass; in the beginning, at 30 minutes contact time, berseem biomass removed 43% (42.62 mg/g) Cu ions. While at 60 minutes, only 20% (20.45 mg/g) Cu biosorption was being observed. But at 90 minutes, 44% (43.92 mg/g) Cu removal was shown. However, at 120 minutes, 49 % (48.56 mg/g) uptake of Cu was noticed. On the basis of above results, it can be noticed that the optimum time for Cu uptake by berseem biomass is 120 minutes. Wheat biomass was acting as a better biosorbent as compared to berseem biomass.

3.2.2. Cd

Figure 2 revealed the biosorption tendency of wheat and berseem biomass at varying time intervals (30, 60, 90 and 120 minutes). The results depict that wheat biomass showed different biosorption potential at different time intervals. Initially, at 30 minutes contact time, wheat biomass removed 79% (78.61 mg/g) Cd ions. While at 60 minutes, 82% (81.89 mg/g) Cd biosorption was being observed. At time interval of 90 minutes, 82% (81.54 mg/g) Cd removal was shown. Similarly, contact time of 120 minutes also showed 82% (81.94 mg/g) uptake of Cd.

On the basis of above results, it is obvious that the optimum time for Cd biosorption by wheat biomass is 60, 90 and 120 minutes; because in this case, equilibrium reaches at 60 minutes and it is maintained up to 90 and 120 minutes time interval. In case of berseem biomass; in the beginning, at 30 minutes contact time, it removed 75% (75.16 mg/g) Cd ions. While at 60 minutes, 76% (76.20 mg/g) Cd biosorption was being observed. At 90 minutes, 75% (74.62 mg/g) Cd removal was shown. However, at 120 minutes, 81% (81.37 mg/g) uptake of Cd was noticed. On the basis of above results, it can be noticed that the optimum time for Cd uptake by berseem biomass is 120 minutes. If comparison of biosorption capacity of wheat and berseem biomass is done while considering contact time then it can be noticed by comparing minimum and maximum biosorption values that wheat biomass is acting as better biosorbent as compared to berseem biomass.

3.2.3. Pb

The results depict that wheat biomass showed different biosorption potential at different time intervals. Initially, at 30 minutes contact time, wheat biomass removed 62% (61.99 mg/g) Pb ions. While at 60 minutes, 71% (70.6 mg/g) Pb biosorption was being observed. 90 minutes time interval also showed 71% (71.83 mg/g) Pb removal. However, contact time of 120 minutes resulted in maximum 81% (80.7 mg/g) uptake of Pb. On the basis of above results, it is obvious that the optimum time for Pb biosorption by wheat biomass is 120 minutes. In case of berseem biomass; in the beginning, at 30 minutes contact time, it removed 68% (68.08 mg/g) Pb ions. While 60 minutes time interval gave maximum 80% (79.62 mg/g) Pb removal. Contact time of 90 minutes showed 72% (72.3 mg/g) Pb removal and at 120 minutes time interval, a little more i.e. 73% (72.5 mg/g) uptake of Pb was noticed. On the basis of above results, it can be noticed that the optimum time for Pb uptake by berseem biomass is 60 minutes. If biosorption capacity of wheat and berseem biomass is compared while considering contact time then it can be noticed by comparing minimum and maximum biosorption values that berseem biomass is acting as better biosorbent as compared to wheat biomass.

3.2.4. Cr

The results depict that wheat biomass showed different biosorption potential at different time intervals. Initially, at 30 minutes contact time, wheat biomass removed 65% (65.12 mg/g) Cr ions. While at 60 minutes, 64% (63.74 mg/g) Cr biosorption was being observed. 90 minutes time interval showed maximum 73% (73.06 mg/g) Cr removal. However, at 120 minutes 57% (56.54 mg/g) uptake of Cr was noticed. On the basis of above results, it is obvious that the optimum time for Cr biosorption by wheat biomass is 90 minutes. In case of berseem biomass; in the beginning at 30 minutes contact time, berseem biomass removed 59% (58.53 mg/g) Cr ions. While at 60 minutes, 64% (64.45 mg/g) Cr biosorption was observed. 90 minutes contact time resulted in maximum Cr removal, 79% (79.04 mg/g). While 120 minutes time interval showed 77% (76.67 mg/g) uptake of Cr. On the basis of these results, it can be noticed that the optimum time for Cr uptake by berseem biomass is 90 minutes. If biosorption capacity of

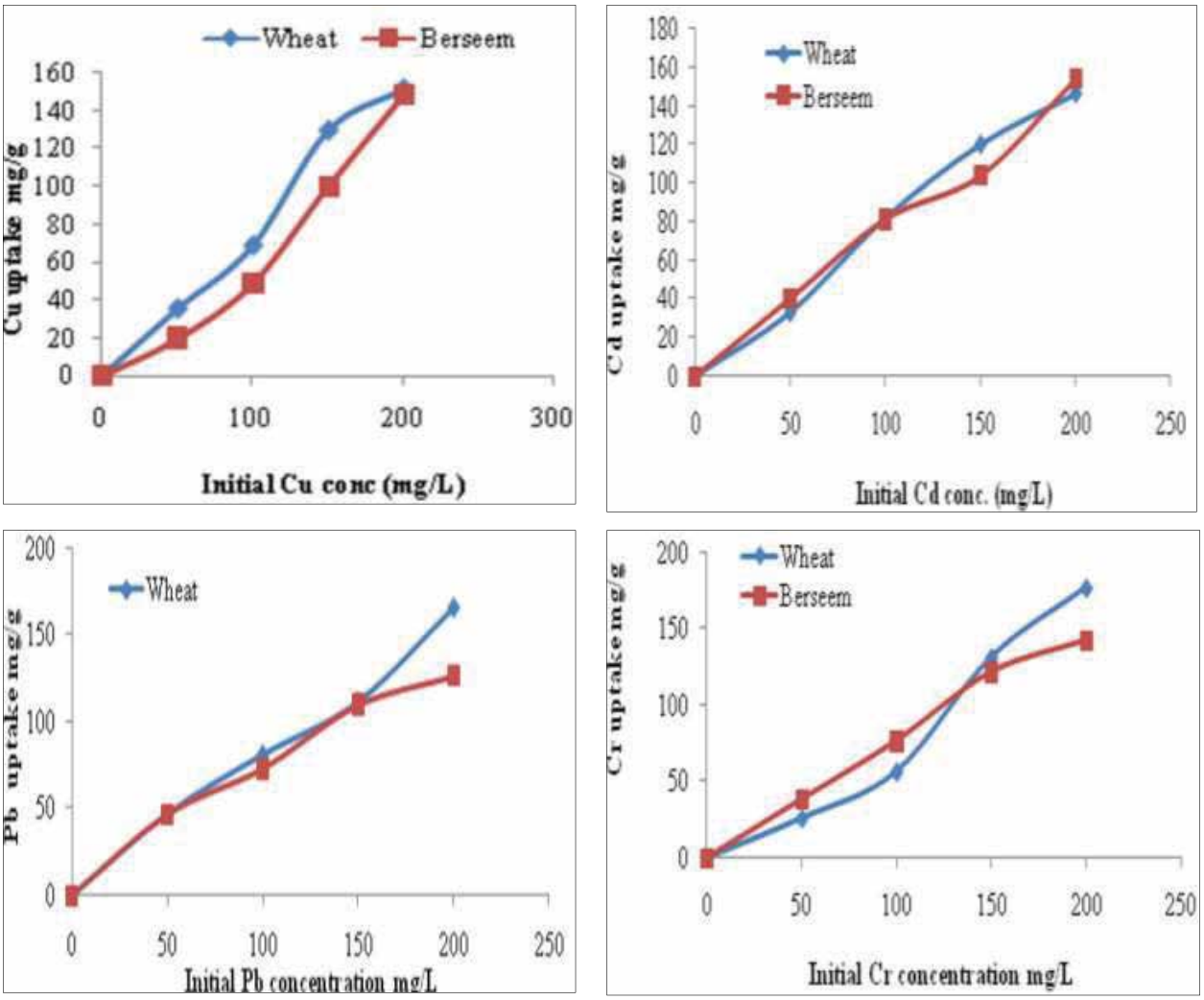

Fig. 1. Effect of initial concentration of metals Cu, Cd, Pb and Cr on the biosorption potential of Wheat and Berseem dry biomass

wheat and berseem biomass is compared while considering contact time; then it can be noticed by comparing minimum and maximum biosorption values that berseem biomass is acting as better biosorbent as compared to wheat biomass.

Optimum contact time for agro based waste material sorption of Pb and Ni was found to be 60 minutes. Finding of this research is similar to finding of this study, which revealed that 60 minutes was optimum contact time for Cu and Cd removal by wheat biomass and 60 minutes was also optimum contact time for Pb removal by berseem biomass. Contact time of 90 minutes was found as optimum time for biosorption of Mn and Pb ions by spinach stalk and pawpaw seed. Contact time of 120 minutes proved to be optimum for biosorption potential of an agro based waste corn cob powder against Cr (VI). This study is similar to results of present study, which revealed that optimum contact time for Cd and Pb uptake by wheat biomass was 120 minute and 120 minutes contact time was also found as optimum contact time for uptake of Cu and Cd uptake by berseem biomass. Zvinowanda *et al.* [25] investigated optimum time observed for metal removal was 120 minutes for biosorption of agro based waste maize tassel for removal of Cd (II) and Cr (VI).

3.3 Effect of pH for biosorption capacity of Wheat and Berseem biomass for metals concentration

3.3.1. Cu

Biosorption of Cu ions by wheat biomass was maximum 69% (68.59 mg/g) at pH value 5 and was minimum 57 % (56.656 mg/g) at pH value 9.

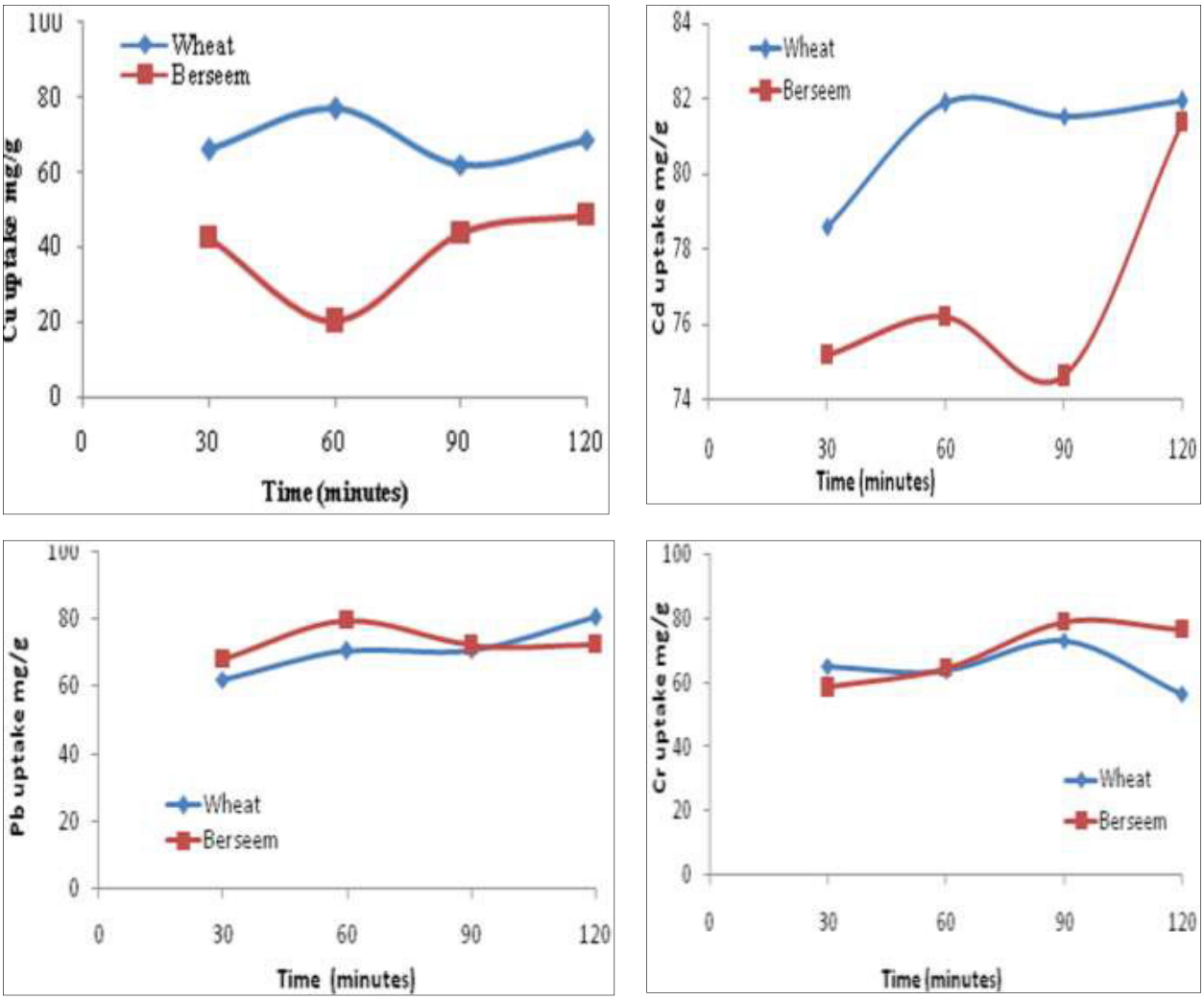

Fig. 2. Effect of contact time on the biosorption of Cu, Cd, Pb and Cr by Wheat and Berseem dry biomass

While at pH value 7, Cu removal percentage was 62% (62.13 mg/g), which is between the other two values. Better Cu removal trend has been shown in acidic medium. This Cu removal trend decreases as the solution turns neutral and it decreases even more in basic solution at pH value 9. Whereas, in case of berseem biomass, biosorption capacity is maximum 79% (78.75 mg/g) at pH value 9 and it was minimum 49% (48.56 mg/g) at pH value 5. It is clear from these results that optimum pH value for Cu removal by berseem biomass is 9. If wheat and berseem biomass are compared in terms of biosorption capacity when varying pH value is conditioned; then it can be noticed by comparing minimum and maximum biosorption values that berseem biomass is acting as a better biosorbent as compared to wheat biomass. Another point to be noticed was that wheat biomass biosorbs Cu ions better in acidic medium whereas berseem biomass biosorbs Cu ions better in basic medium.

3.3.2. Cd

The biosorption of Cd ions by wheat biomass was maximum 82% (81.94 mg/g) at pH value 5 and minimum 61% (61.15 mg/g) at pH value 9. While at pH value 7, Cd removal percentage was 80% (79.97 mg/g) that is between the other two values. Better Cd removal trend has been shown in acidic medium. This Cd removal trend decreases as the solution turns neutral and it decreases even more in basic solution at pH value 9. Whereas, in case of berseem biomass, biosorption capacity is maximum 82% (81.7 mg/g) at pH value 9 and it is minimum 80% (80.43 mg/g) at pH value 7. While at pH value 5, 81% (81.37 mg/g) Cd removal is between the other two values. It is clear from these results that optimum pH value for Cu removal by berseem biomass is 9. In case of berseem biomass, this Cd uptake potential is slightly more in basic medium.

3.3.3. Pb

Biosorption of Pb ions by wheat biomass was maximum 87% (86.89 mg/g) at pH value 9 and minimum 32% (32.45 mg/g) at pH value 7. While at pH value 5, Pb removal percentage was 81% (80.7 mg/g), which is between the other two values. These results depicted that optimum pH value for Pb uptake by wheat biomass is 9. Better Pb removal trend has been shown in basic medium. This Pb removal trend decreases as the solution turns neutral but it increases again in basic solution at pH value 5. Whereas, in case of berseem biomass, biosorption capacity is maximum 82% (81.58 mg/g) at pH value 9 and it is minimum 44% (44.19 mg/g) at pH value 7. While at pH value 5, 73% (72.51 mg/g) Pb removal is between the other two values. It is clear from these results that optimum pH value for Pb removal by berseem biomass is 9. Like wheat biomass, berseem biomass also shows better Pb removal trend in basic medium. If wheat and berseem biomass are compared as biosorbents in terms of biosorption capacity when varying pH value is conditioned then it can be noticed by comparing minimum and maximum biosorption values that wheat biomass is acting as better biosorbent as compared to berseem biomass.

3.3.4. Cr

Figure 3 shows that biosorption of Cr ions by wheat biomass was maximum 68% (68.37 mg/g) at pH value 9 and minimum 50% (49.87 mg/g) at pH value 7. While at pH value 5, Cr removal percentage was 57% (56.54 mg/g), which is between the other two values. These results depict that optimum pH value for Cr uptake by wheat biomass is 9. Better Cr removal trend has been shown in basic medium. Whereas, in case of berseem biomass, biosorption capacity is maximum 79% (78.86 mg/g) at pH

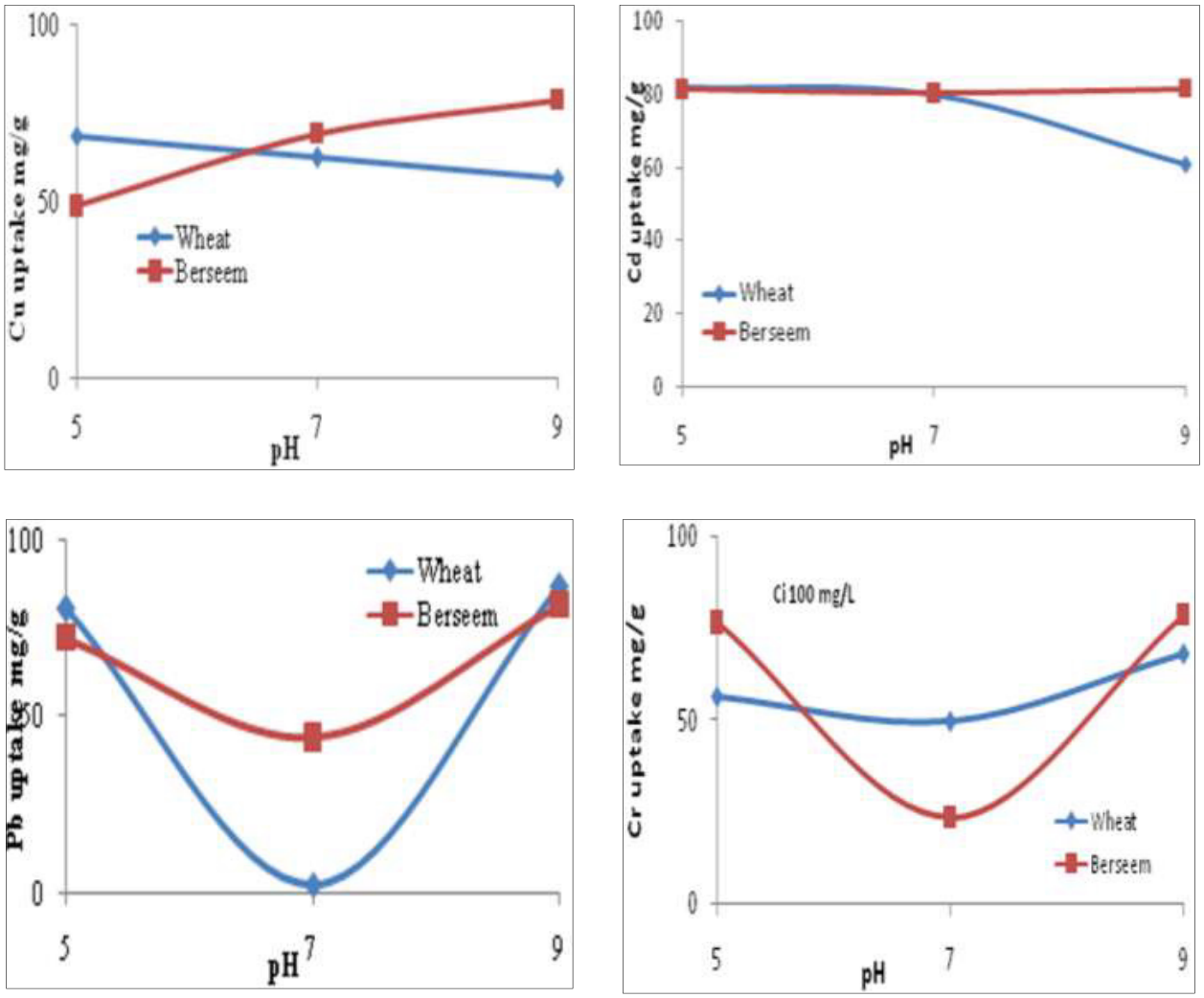

Fig. 3. Effect of pH on the biosorption of Cu, Cd, Pb and Cr by Wheat and Berseem dry biomass

Table 1: Kinetic modelling expressing regression coefficient R2 of biosorption by wheat and berseem biomass via Langmuir and Freundlich isotherm

	Langmuir isotherm								Freundlich isotherm							
	Wheat biomass				Berseem biomass				Wheat biomass				Berseem Biomass			
	Cu	Cd	Pb	Cr	Cu	Cd	Pb	Cr	Cu	Cd	Pb	Cr	Cu	Cd	Pb	Cr
pH	0.99	0.99	0.99	0.99	0.98	1	0.99	0.99	0.99	0.99	0.96	0.98	0.96	0.99	0.96	0.99
Contact time	0.99	1	0.99	0.99	0.99	0.99	0.99	0.99	0.98	0.99	0.96	0.98	0.98	0.99	0.98	0.98

value 9 and it is minimum 24% (23.55 mg/g) at pH value 7. While at pH value 5, 77% (76.67 mg/g) Cr removal is between the other two values. It is clear from these results that optimum pH value for Cr removal by berseem biomass is 9. In contrast to wheat biomass, better Cr removal trend has been shown in both acidic and basic medium. In case of berseem biomass, this Cr uptake behaviour decreases as the solution turns neutral. Another point to be noticed is that wheat biomass biosorbs Cr ions better in acidic medium whereas berseem biomass biosorbs Cr ions with almost equal efficacy in both acidic and basic mediums. It has been generally agreed that metal removal intensity of biosorbents can be strongly influenced by pH value of metal solution indicating that the process is governed by an ion-exchange method. At high acidic pH values of the solution, there is increased concentration of hydrogen (H^+) and hydronium (H_3O^+) ions, which compete efficiently with metal ions in binding to negatively charged groups on the biosorbent surface. Therefore, enhancement in heavy metal removal with increasing pH values could be credited to less ionic competition [14].

A number of researchers have also investigated the effect of pH value on biosorption of heavy metals by using different biomass and found similar results with the this study. Han *et al.* [26] used cereal chaff as a biosorbent for Pb and Cu adsorption and found that there was an increase in the Pb uptake when the pH value of the medium was increased and had optimum pH value ranging 5-6 but maximum pH value for Cu and Pb uptake was 9. These findings are similar to the findings of the current study where maximum biosorption was attained at pH value of 9 by berseem biomass against all the four metals under consideration (Cu, Cd, Pb and Cr). Whereas maximum biosorption was also attained at pH value 9 by wheat biomass against Pb and Cr. Agro based by product *Tamarindus indica* seeds (Indian date) also used as biosorbent for uptake of Cr (VI) and applied at pH range 4-9. Maximum metal removal was observed at pH 9. Wheat shell biomass act as biosorbent for Cu (II) from aqueous solutions and adsorption capacity was maximum at pH value 5. Metal chemistry and sorbent surface binding sites are influenced by the pH value and sorption increased at pH 5 [27]. Agricultural waste olive pomace has biosorption potential against Pb, Cd and Cu and has pH influenced for metal ions uptake and maximum biosorption been achieved at pH 5 for all three metals [28-33].

3.4 Statistical analysis

Biosorption of Cu, Cd, Pb and Cr were investigated for kinetic parameters via Langmuir and Freundlich isotherm that expressed varying degrees of sorption (Table 1). Both the models fitted well with isotherms applied however, $R^2 = 1$ was obtained for Cd biosorbed by both wheat and berseem biomass that signifies the perfect fitting of data in Langmuir model. The adsorptive interactions indicated by both isotherms developed between metals and biosorbents can either be due to transfer of metallic mass over the boundary or the metallic ions can also be sorbed onto the biomass material. However there are chances of intra-particle diffusion as well. Either mechanism occurring for metals removal via wheat and berseem biomass marks the potential of these biosorbents in pollution remediation via facile, cost effective and eco-friendly mode. Such favourability of biosorbents is also indicated by the linearity.

4. CONCLUSION

Heavy metals contaminated soils can be sustainably remediated by utilization of plant varieties that

are inherently provided with the ability of heavy metal uptake. The positive results of the current research for heavy metal i.e. copper (Cu), cadmium (Cd), lead (Pb) and chromium (Cr) removal via Wheat (*Triticum aestivum*) and Berseem (*Trifolium alexandrinum*) biomass is indicative of the future prospects of these plants in environmental cleanup. Phytoremediation done via Wheat and Berseem plant is not only an environmental friendly strategy but it also serve as an economical alternative to the conventional physicochemical methods of remediation. Energy crops after heavy metal uptake can be used as a biodiesel and estimations regarding its effectiveness can be done.

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