International Journal of Engineering, Science and Mathematics
Vol. 6 Issue 6 , October 2017,
ISSN: 2320-0294 Impact Factor: 6.765
Journal Homepage: http://www.ijesm.co.in, Email: ijesmj@gmail.com
Double-Blind Peer Reviewed Refereed Open Access International Journal - Included in the International Serial Directories Indexed & Listed at: Ulrich's Periodicals Directory ©, U.S.A., Open J-Gage as well as in Cabell's Directories of Publishing Opportunities, U.S.A

The Assessment of Morphological-Biochemical Changes and the Bioactivity of Low Molecular Weight Peptide(s) isolated from Waste Water Treated Mung Bean (*Vigna radiata*) Plant

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Abstract

Waste water is important for plants for its growth and development and its use as irrigational source is very common in countries like India. In this study, waste water from outfalls of river Ganga in West Bengal region were analyzed season wise during the period of 2016. The effect of waste water was measured morphologically and biochemically on Mung Bean (*Vigna radiata*). An approach to study the range of low molecular weight peptide(s) (3-0.5 kDa) were taken through its isolation by solvent extraction method, ultra filtration and HPLC both in normal and treated plants.

The study during pre-monsoon and post monsoon season indicated by stem length, leaf length, lead width, petiole length and intermodal distance were found inhibited by waste water treatment. Whereas, the isolated peptide(s) induced by waste water treatment showed the inhibition of Mung Bean (*Vigna radiata*)seed germinations. The inhibition was found pronounced in premonsoon and post monsoon in comparing to monsoon season.

Keywords:

Waste water
Mung Bean (*Vigna radiata*);
Low molecular weight peptide(s);
Seed germination

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1. Introduction

River Ganga is subjected to multiple uses for community water supply, irrigation, bathing, and disposal of sewage and industrial effluents. Another report highlights more than half of India's river and other surface water bodies are significantly polluted. Supreme Court of India (September, 1987) ordered the tanneries of Kanpur to either clean their waste or shut down before release of waste in river Ganga. Econometric Model was used to mention the effects on pollution and infant mortality at various districts of Kanpur [1]. Due to increased human population, industrialization, use of fertilizers and man-made activity water is highly polluted with different harmful contaminants. It is necessary that the quality of drinking water should be checked at regular time interval, because due to use of contaminated drinking water, human population suffers from varied of water borne diseases. It is necessary to know details about different physico-chemical parameters such as colour, temperature, acidity, hardness, pH, sulphate, chloride, DO, BOD, COD, alkalinity used for testing of water quality. Heavy metals such as Pb, Cr, Fe, Hg etc. are of special concern because they produce water or chronic poisoning in aquatic animals. Due to use of contaminated drinking water, human population suffers from varied of water borne diseases [2]. A questionnaire-based survey was used to estimate water-borne and enteric disease incidence and study river use among resident users of the Ganges River in Varanasi. The overall rate of water-borne/enteric disease incidence, including acute gastrointestinal disease, cholera, dysentery, hepatitis-A, and typhoid, was estimated to be about 66% during the one-year period prior to the survey [3]. To investigate pollution status of Gangetic river system of Uttarakhand, India, physicochemical and microbiological analysis of river water collected from 32 different sites were performed season wise. The analysis includes total viable count, total coliform count and faecal streptococcal count. pH, temperature, specific conductance, total dissolved solids (TDS), dissolved oxygen (DO), biological oxygen demand (BOD) and chemical oxygen demand (COD). It is useful as an indictor of water quality of this region. The bacterial genera were identified on the basis of their morphological and physiological characteristics and confirmed the presence of bacterial indicators of faecal origin at various altitudes in every stretch of Gangetic river system which pointing towards an alarming situation [4]. A hydro-chemical study was carried out in River Ganga from Deoprayag to Rishikesh (India) sediment and nutrient load has been considered to evaluate the current state of pollution through real time measurements. The pH and conductance were within the limits prescribed for drinking water. The maximum suspended sediment concentrations and nutrient load found in rainy season and concluded that in a mountain environment with low agricultural activities, the stream chemistry is greatly influenced by the agricultural practices carried out on the terraced land were the source [5]. Water samples from three sewage treatment plants which regularly discharge into the River Ganga were analyzed at Varanasi. The most probable number index of *E.coli* in water samples and coliform counts were recorded as being higher in irrigated water samples and vegetables, indicating a serious health hazard posed by intense microbial and faecal pollution. BOD, DO Heavy metals (Zn, Cu, Cd, Pb, Cr) in disposed effluents were above permissible limits at all three sites [6]. Photosynthetic activity plays an important role in maintaining high levels of DO in Ganga. An assessment on photosynthetic activity and oxygen production rates in the river and its correlation with various water quality parameters. In the entire Kannauj-Kanpur stretch both BOD and DO levels in the river has increased, except at Jajmau, where anaerobically treated effluent is discharged to the river [7]. Another study on river Ganga in Haridwar District the determination of water quality index considering temp, velocity, pH, dissolved oxygen, free CO_2, C.O.D., B.O.D., Carbonate, Bicarbonate, total alkalinity, hardness, turbidity, calcium, magnesium, sodium, potassium, nitrate, phosphate, chloride, sulphate, electrical conductivity, total dissolved solids and total suspended solids [8]. The physico-chemical parameters study of Kosi River shows the parameters are not within the standard value and it was concluded that the river water is not suitable for dinking and domestic purpose. Fluoride content in water is found in the range of 0.32-1.7 ppm which can lead to dental and skeleton problems in the peoples of the areas [9].The use of waste water for irrigational purposes is well known for the countries like India. Again, its impact on different plants had been observed in different aspects. *Vigna radiata* is a common edible plant in India is used for various study regarding this purpose. A study in UP, phytotoxic and genotoxic effects on Mung Bean (*Vigna radiata)* had been investigated by using treated and untreated tannery waste water. Seeds of Mung Bean were treated for 15 days with different concentration of tannery waste water. A significant reduction in seed germination had been found when treated with 25% untreated effluent and 75% treated effluent, compared to control. Even the growth of the plants was inhibited. RAPD profiles were obtained for selected Mung Bean (*Vigna radiata)* plants where the appearance and disappearance of new bands were significant for both untreated and treated effluent cases [10]. A comparative growth response on two varieties of Mung Bean (*Vigna radiata)*(var. PDM 54 and var. NM 1) was found when they were treated with tannery wastewater and ground water. The increase of plant biomass for the use of tannery wastewater was found where no significant changes were notes for its biochemical properties like protein and chlorophyll content. In case of both varieties up to use of 35% tannery waste water shows positive growth parameters [11]. Another study on effect of raw distillery effluent and the post-treatment effluent showed percentage of germination, speed of germination index, vigor index and length of root and embryonic axis deceasing for raw distillery effluent on Mung Bean (*Vigna radiata*) [12]. Free radicals are responsible for many diseases in human being. Antioxidant components help to minimize those diseases [13]. For antioxidant based defense system, the inhibitory response of proteins was found significant [14]. A study on low molecular weight peptide(s) (0.5 to 3 kDa) focused to determine the antioxidant potential of peptide(s) isolated from Mung Bean (*Vigna radiata*). It was found that LMW peptide(s) fractions had higher DPPH scavenging activities than HMW peptide(s) [15]. In between 0.5-3 kDa, similar results were obtained for mulberry plants [16].

The study aims to identify major domestic outfalls in River Ganga in West Bengal region. A section of the river was selected to identify major outfalls in both east and west bank. A thorough survey and analysis highlighted a major outfall near Dakhinewar Ferry Ghat, West Bank of River Hooghly, North 24 PGS and India which was selected for the further studies. The impact of waste water collected from that selected outfall had been observed on Mung Bean (*Vigna radiata*).

2. Research Method

2.1 Collection of waste water and its analysis:
The selected outfall of West Bengal near Dakhineswar ghat (N22^{0}39’13.2’’ E088^{0}21’27.1’’) was identified for the collection of waste water in three consecutive seasons of the year 2016. The analysis of its physical, chemical, and biological parameters was done in premonsoon, monsoon and post monsoon season [17]. The samples were taken carefully in the laboratory and stored in proper condition. Parameters like DO, pH, temperature were analyzed immediately in the field using proper methods and instruments. Other parameters were analyzed in the laboratory within collection of 7-10 days of the sample.

2.2 Selection of Plant and Treatment of waste water and plant peptide(s) on seed germination:
The edible seeds of Mung Bean (*Vigna radiata*)familyLeguminosae (Fabaceae) were collected from local market for the experimental purposes. The effect of collected waste water and peptide(s) extract from treated plants on seed germination of Mung Bean (*Vigna radiata*)was observed. 0.01% Mercuric chloride was used for 5 min to remove microbes. By using following the standard methods [18] used by P. Bhattacharya et.al the germination percentage was calculated for the both cases.
Germination percentage = No. of seeds germinated / Total no of seeds x 100

2.3 Study of Morphological and Biochemical Parameters of Waste water treated and untreated plants:
Garden soil was used to grow Mung Bean (*Vigna radiata*)Plants for the each season. Plants were grown for 10 days in controlled condition then divided into 2 sets. Each sets contained 10 plants which were exposed to waste water and tap water treatment separately. Parameters like stem length, leaf length, lead width, petiole length, and internodal distance were measured in three days intervals upto 15 days. The biochemical parameters like chlorophyll and protein content were estimated by using standard methods [19]-[20].

2.4 Solvent Extraction:
All plant materials treated and untreated were cleaned properly. Separately they were weighted in an electronic balance. 10 gm of each type blended in a mortar and pestle with distilled water (1:10 w/v) for further biochemical analysis. The filtrate materials of aqueous plant extracts was stored in refrigerator at -20^{0}C for further study.

2.5 The HPLC chromatogram analysis of semi purified peptide(s) (3-0.5 kDa):
10 gm of Mung Bean (*Vigna radiata*) seeds treated and untreated (for premonsoon season) were grinded by using mortar and pestle in the presence of phosphate buffer saline (pH 7.4). The centrifugation of the prepared sample was done at 12000 rpm maintaining 4^{o}C for 15 minis. The supernatant was collected and filtered by 100 µm Millipore Filter. For peptide(s) analysis, the extract was mixed with methanol (1:1 v/v). By using Rotary Evaporator at 65 ^{o}C the mixture was treated for several minutes until it was reduced to 5-10 ml [18]. After

discarding anionic hormones the extract were filtered through Millipore ultra filtration systems by following the standard methods used by Ghosh *et. al.* (2010) and Bhattacharya *et.al* (2014)[18], [21].

Using HPLC methods a range of peptide(s) 3-0.5 kDa were isolated for treated and non treated plants. The effect of isolated low molecular weight peptide(s) on seed of Mung Bean (*Vigna radiata*)had been observed.

3. Results and Analysis

3.1 The Analysis of Physico-Chemical and Microbial Parameters:
The water quality of selected outfall of river Ganga had been analysed on the basis of its Physico-Chemical and Microbial Parameters [Table 1]. According to Environmental Protection Rules, 1986 [22], the permissible limit of pH of inland surface water is 5.5 to 9.0. The values of pH were varied from 6.8 to 6.9 in every season nearly in neutral condition. Season wise a significant variation of temperature was observed. During post monsoon it was reported 19°C, where as in premonsoon and monsoon period it prevailed within 28-29 °C. According to Central Pollution Control Board (CPCB), the permissible limit of DO for outdoor bathing was minimum 5.0 mg/L. For fisheries and wildlife propagation, the value is minimum 4.0 mg/L but for every season it came 0 or nearly 0. According to CPCB, the permissible limit of conductivity of water for propagation of wildlife and fisheries was 1000 mS/cm at 25^0C. Season wise analysis showed for every season it had crossed the standard. The permissible limit of BOD in Inland surface water is 30 mg/L whereas the permissible limit of BOD in public sewers is 350 mg/L according to Environmental Protection Rules, 1986, BOD value found within the permissible limit for each case.

Table 1: physico-chemical and microbial parameters of selected outfall

Parameters	**Values**		
	PRE-MONSOON	**MONSOON**	**POST MONSOON**
pH	6.8	6.8	6.9
Temp (°C)	29	28	19
DO (mg/L)	0	0.47	0
Conductivity 25 °C (mS/cm)	1768	1328	1491
BOD (mg/L)	7	4.6	6
Nitrate Nitrogen (mg/L)	33	20.2	19.2
Chloride (mg/L)	158.32	72.86	120.1
Hardness (mg/L)	286	270.2	276.2
Phosphate (mg/L)	31	19	22
As (mg/L)	0.002	0.001	0.002
Hg (mg/L)	0.05	0.03	0.04
Pb (mg/L)	0.007	0.004	0.006
Total Coliform Count, MPN/100 mL	2.9×10^{7}	1.8×10^{7}	2.7×10^{7}
Fecal Coliform, MPN/100 mL	4.7×10^{6}	2.4×10^{6}	4×10^{6}

The permissible limit of Nitrate-nitrogen in Inland surface water is 10 mg/L, it was found little higher for every season. The obtained concentration of Chloride did not cross the permissible limit of it given by CPCB in Inland surface water which is 250 mg/L when this water is used as drinking water without conventional treatment but after disinfection. No such permissible limit of hardness in inland surface water was found from CPCB or Environmental Protection Rules, 1986. So, it is not clear the maximum permissible limit of hardness in Inland surface water. According to Environmental Protection Rules, 1986, the permissible limit of soluble phosphate (as P) is 5 mg/L. This study reported the range of it 19-33 mg/L. The presence of heavy metal like As (mg/L), Hg (mg/L), Pb (mg/L) did not show any significant result. The range of Total Coliform Count, MPN/100 mL and Fecal Coliform, MPN/100 mL were found very high and significant for each season compared to CPCB standard limit i.e 500 or less for outdoor bathing [23].

Table 2: Season wise changes of Morphological Parameters of Mung Bean (*Vigna radiata*)

DAYS	**STEM HEIGHT (cm)**					
	PREMONSOON		**MONSOON**		**POSTMONSOON**	
	CONTROL	TEST	CONTROL	TEST	CONTROL	TEST
DAY1	8.67	8.55	8.65	9.00	8.73	8.70
DAY3	8.83	8.55	8.88	9.20	8.95	8.58
DAY6	9.00	8.47	9.25	9.47	9.21	8.26
DAY9	9.13	8.46	9.60	9.67	9.44	7.84
DAY12	9.36	8.51	10.76	10.52	9.76	7.47
DAY15	9.67	8.53	11.76	11.18	10.07	7.01
DAYS	**LEAF LENGTH (cm)**					
DAY1	3.07	3.05	3.07	2.97	3.10	3.15
DAY3	3.26	3.11	3.23	3.13	3.30	3.13
DAY6	3.42	3.10	3.42	3.37	3.52	2.94
DAY9	3.57	3.10	3.60	3.46	3.81	2.90
DAY12	3.75	3.11	3.96	3.78	3.99	2.77
DAY15	3.95	3.11	4.24	4.07	4.15	2.76
DAYS	**LEAF WIDTH (cm)**					
DAY1	1.16	1.14	1.16	1.14	1.16	1.14
DAY3	1.20	1.13	1.16	1.14	1.15	1.13
DAY6	1.21	1.10	1.19	1.17	1.28	1.09
DAY9	1.25	1.10	1.22	1.18	1.33	1.09
DAY12	1.27	1.11	1.26	1.23	1.38	1.04
DAY15	1.31	1.11	1.31	1.26	1.42	1.03
DAYS	**PETIOLE LENGTH (cm)**					
DAY1	0.13	0.18	0.13	0.15	0.13	0.18
DAY3	0.16	0.16	0.13	0.15	0.16	0.16
DAY6	0.18	0.14	0.14	0.16	0.18	0.14
DAY9	0.21	0.11	0.14	0.16	0.24	0.11
DAY12	0.25	0.10	0.16	0.18	0.29	0.10
DAY15	0.26	0.10	0.17	0.19	0.33	0.10
DAYS	**INTERNODAL DISTANCE (cm)**					
DAY1	2.40	2.31	2.42	2.39	2.44	2.48
DAY3	2.40	2.36	2.42	2.43	2.51	2.44
DAY6	2.43	2.41	2.48	2.48	2.57	2.27
DAY9	2.44	2.43	2.48	2.48	2.63	2.11
DAY12	2.52	2.47	2.74	2.69	2.72	2.03
DAY15	2.60	2.54	2.92	2.88	2.83	2.03

The morphological study of Mung Bean (*Vigna radiata*)plants by measuring the parameters like stem height, leaf length, leaf width, petiole length and internodal distance were done in 3 days interval of starting of the experiment. The result of average values of each set of plants for each parameter showed the most observable negative impact on plants growth during pre monsoon period in compared to control and other seasonal analysis [Table 2; Figure1-5].

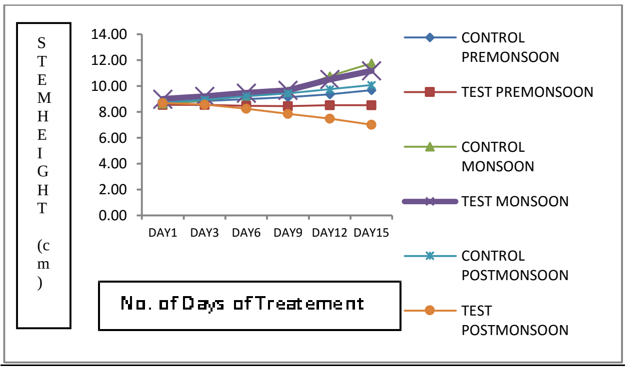

Figure1: Day wise changes of STEM HEIGHT (cm) of *Vigna radiata* in Control and Test plants for three seasons

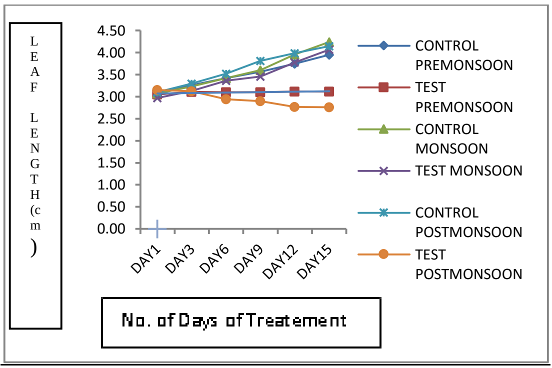

Figure2: Day wise changes of LEAF LENGTH (cm) of *Vigna radiata* in Control and Test plants for three seasons

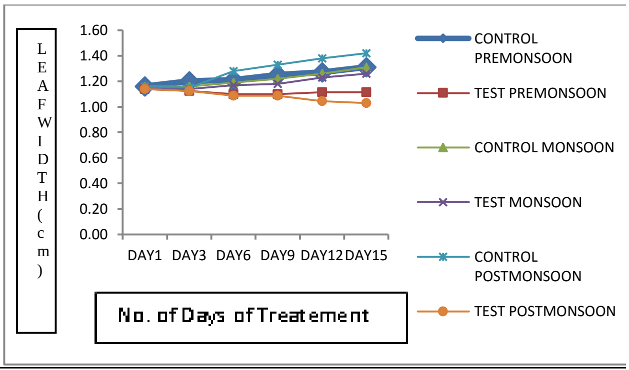

Figure3: Day wise changes of LEAF WIDTH (cm) of *Vigna radiata* in Control and Test plants for three seasons

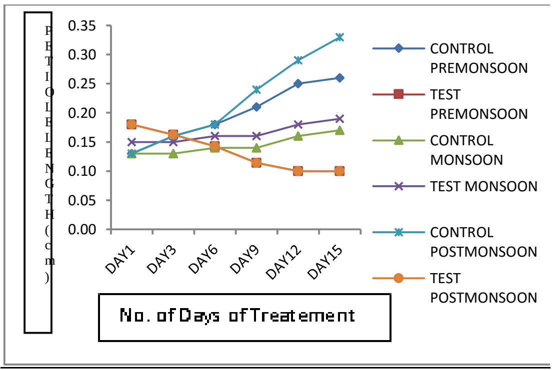

Figure4: Day wise changes of PETIOLE LENGTH (cm) of *Vigna radiata* in Control and Test plants for three seasons

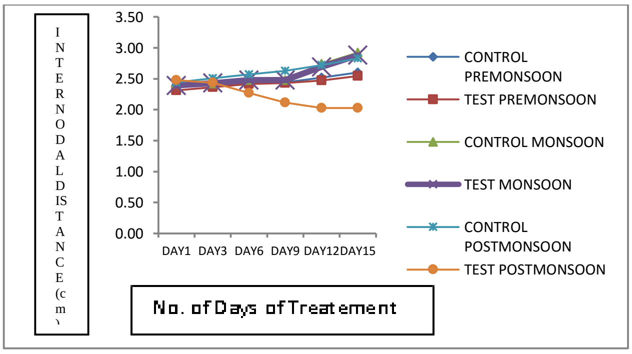

Figure5: Day wise changes of INTERNODAL DISTANCE (cm)of *Vigna radiata* in Control and Test plants for three seasons

The study of protein content for both control and treated plant showed a reduction tendencey in every season. For control plant the reduction percentage after 15 days were 5.49%, 3.41% and 6.67% in premonsoon, monsoon and postmonsoon period respectively, wehere as in treated plants the percentage of reduction was found significantly high i.e 42.86%, 11.36% and 24.72% season wise. In case of chlorophyll content of control plants the percentage was slightly increased i.e 1.79%, 1.64% and 0% in premonsoon, monsoon and postmonsoon period respectively. Treated plants analysis showed a decrease of 28.57% for premoonson, 11.48% for monsoon and 22.41% in post monsoon period [Table 3].

Table 3: Season wise changes of Protein and Chlorophyll Content of Mung Bean (*Vigna radiata*)

Season	Protein Content (mg/L)			
	CONTROL (0 DAYS)	CONTROL (15 DAYS)	TEST(0 DAYS)	TEST(15 DAYS)
PREMONSOON	0.91	0.86	0.91	0.52
MONSOON	0.88	0.85	0.88	0.78
POSTMONSOON	0.9	0.84	0.89	0.67
Season	**Chlorophyll (mg/gm of Fresh weight)**			
PREMONSOON	1.12	1.14	1.12	0.8
MONSOON	1.22	1.24	1.22	1.08
POSTMONSOON	1.16	1.16	1.16	0.9

Table 4: The effect of peptide(s) solution (3-0.5kDa) extracted from treated plants on seeds of *Vigna radiata*

Season	% of Germination	
	Control	Treatment
Premonsoon	97%	0%
Monsoon	99%	25%
Postmonsoon	97%	2%

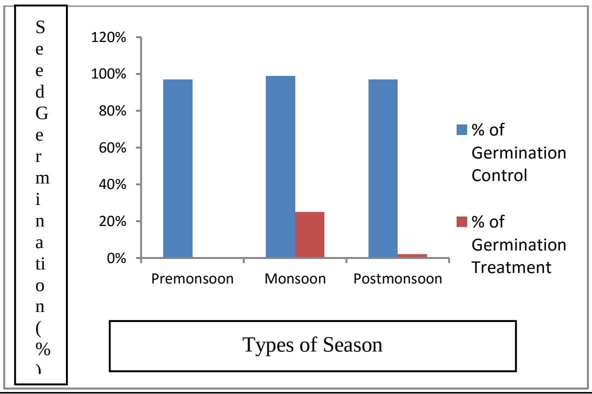

Figure 6: The effect of peptide(s) solution (3-0.5kDa) extracted from treated plants on seeds of *Vigna radiata*

Table 5: The effect of Tap water and Waste water on seed germination

Season	Volume of Tap Water (ml)	Volume of Waste water (ml)	% of Germination
Premonsoon(Control)	10		90
Premonsoon (Test)		10	30
Monsoon (Control)	10		90
Monsoon (Test)		10	60
Postmonsoon(Contr)	10		90
Postmonsoon (Test)		10	40

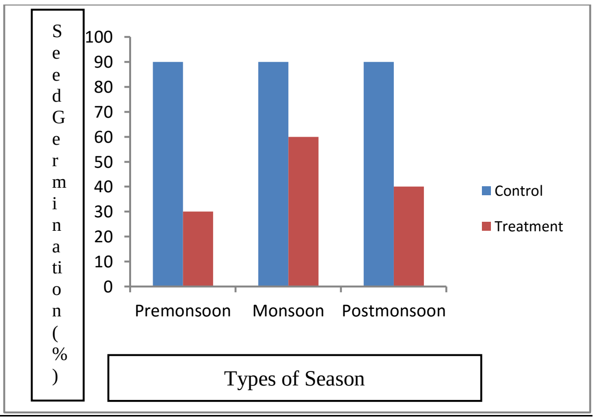

Figure 7: The effect of Tap water and Waste water on seed germination (percentage)

Not only on Mung Bean (*Vigna radiata*) plant, this study had extended to observe the effect of waste water and treated plant extract on seeds of Mung Bean (*Vigna radiata*)[Table 4-5; Figure 6-7]. The results showed for both cases the percentage of germination of seeds had been changed in compared to control. When peptide(s) solution (3-0.5kDa) extracted from treated plants directly applied to seeds, the percentage of germination reduced to 0% during premonsoon. In case of control 97-99% seeds were germinated. Again the effect was more pronounced in premonsoon season too when waste water was applied on seeds. In this case of control sample 90% germinationpercentage was recorded, where as it was reduced to 30%, 60% and 40% during premonsoon, monsoon and post monsoon period respectively. So, for seeds peptide (s) analysis the seeds of premonsoon (treated and untreated) were selected. On the basis of seed germination percentage result, the treated and untreated seeds of premonsoon were selected for further peptide(s) analysis.

S A M P L E   I N F O R M A T I O N

Sample Name:	cm	Acquired By:	System
Sample Type:	Unknow n	Sample Set Name:	
Vial:	1	Acq. Method Set:	Analysis
Injection #:	6	Processing Method:	cm
Injection Volume:	20.00 ul	Channel Name:	486
Run Time:	20.0 Minutes	Proc. Chnl. Descr.:	

Figure 8: Sample information of HPLC chromatogram (Control Mung Bean seed)

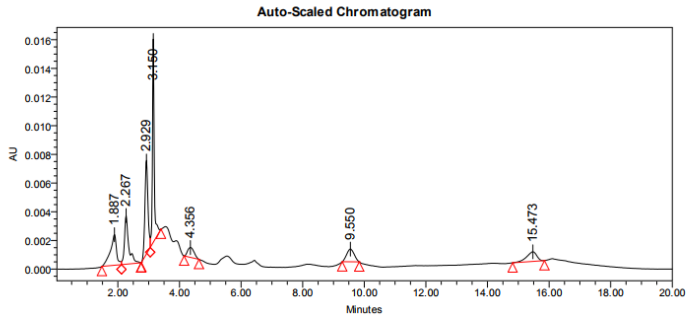

Table 6: Result of Peak Analysis (Control Seed)

SL NO.	RT	AREA	HEIGHT
1	1.887	29841	2138
2	2.267	32348	3383
3	2.929	44204	6413
4	3.15	57237	13408
5	4.356	10197	688
6	9.55	15030	887
7	15.473	15112	666

Figure 9: Auto-Scaled Chromatogram of Mung Bean seed (Control)

S A M P L E   I N F O R M A T I O N

Sample Name:	tm	Acquired By:	System
Sample Type:	Unknow n	Sample Set Name:	
Vial:	1	Acq. Method Set:	Analysis
Injection #:	7	Processing Method:	tm
Injection Volume:	20.00 ul	Channel Name:	486
Run Time:	20.0 Minutes	Proc. Chnl. Descr.:	

Figure 10: Sample information of HPLC chromatogram (Test Mung Bean seed)

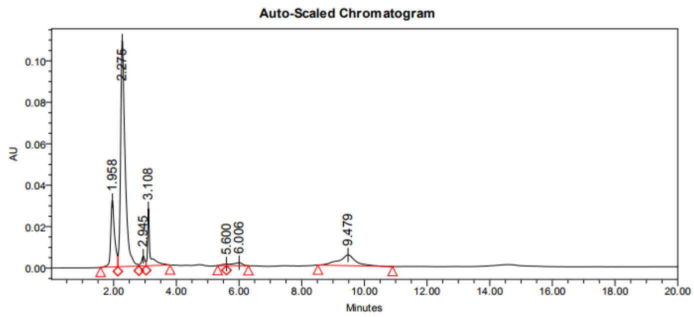

Figure 11: Auto-Scaled Chromatogram of Mung Bean seed (Test)

Table 7: Table 5: Result of Peak Analysis (Test Seed)

SL NO.	RT	AREA	HEIGHT
1	1.958	273551	32393
2	2.275	1088342	110291
3	2.945	34751	4672
4	3.108	155130	26636
5	5.6	7143	737
6	6.006	35154	1474
7	9.479	196200	5192

In control seed peptide(s) were analyzed in HPLC chromatogram and found the appearance of 7 peaks [Table 6; Figure 8-9]. Under waste water treatment the chromatogram exhibits same number of peaks of the peptide(s) where the retention time (RT) analysis showed different pictures [Table 7; Figure 10-11]. Here the peak of retention time of 15.473 min was disappeared and the peak of 4.356 min completely fragmented to yield two peaks of 5.6 and 6.06 min.

Other interesting features about the two peaks are 2.929 min and 3.150 min were reduced by their peak area whereas the peak of 2.267 min was increased in area and expressed few seconds earlier. The picture of the chromatogram exhibited the effect of waste water which is responsible for the synthesis and degeneration of peak of peptide(s) which clearly shows the effect of waste water.

4. Conclusion

After thirty years of launching the first phase of Ganga Action Plan, the status of River Ganga is still at a risk. A significant amount of untreated waste enters into the river in daily basis. The accumulation of microbial density as well as other aquatic pollutants should be a matter of great concern. The negative impact of those untreated wastewater on ecosystem is also very alarming. The study helped to explore the effect of waste water on *Vigna radiata*. The peptide (s) systhesized by waste water treatment even showed a negative effect on its seed germination sesason wise. Premonsoon waste water was found to create more impact than other two. The detailed analysis of peptide(s) even explored a great variation of peak results for treated plant in compared to control of premonsoon. The assorted data will definitely help to take some remedial measures to reduce its pollution load by eliminating the major sources and save River Ganga. With waste full water of Ganga, the morphological parameters of selected plant species are very much subjected under stress.

Acknowledgement

Authors thank Department of Civil Engineering, Jadavpur University and Department of Environmental Science, Asutosh College for their laboratory support.

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