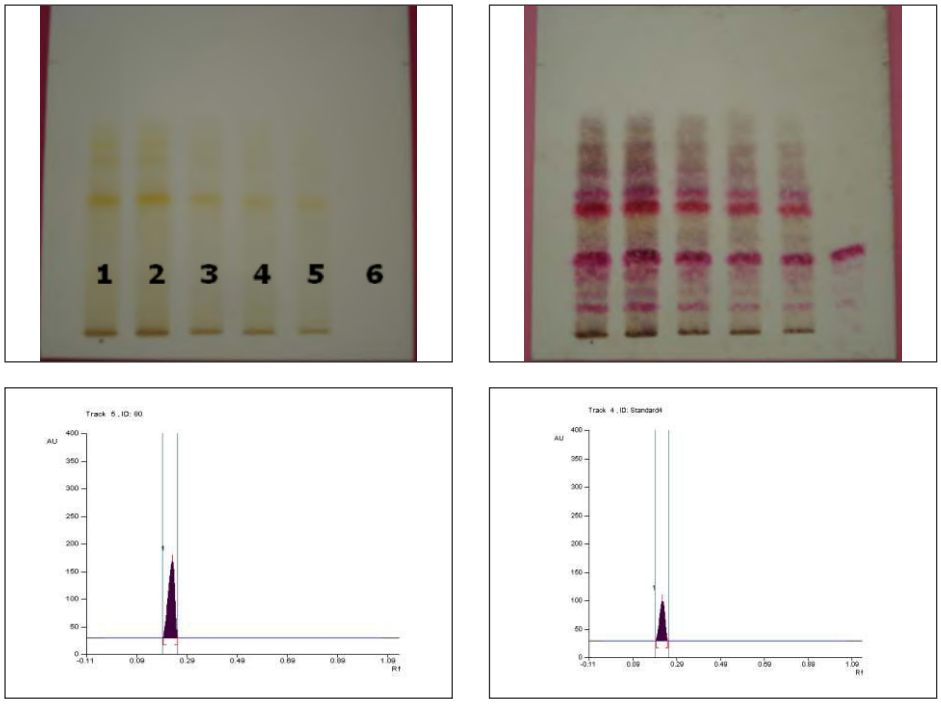
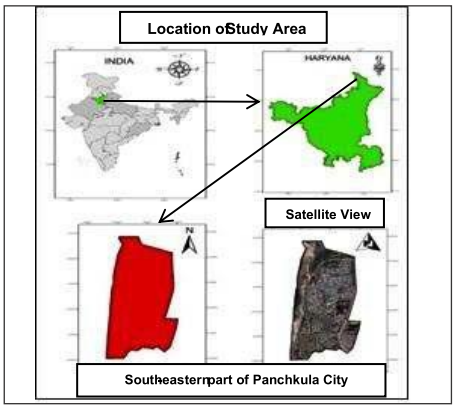
Volume 2, Issue 3 - July - September, 2020

ISSN : 2582-5283
DOI: 10.47062
Indexed in International Scientific Indexing

International Journal of Environment and Health Sciences



Standard Sample



SAVE THE ENVIRONMENT (STE)
Chief Editor: Dr. Kshipra Misra
Phone: +91-9871372350 • E-mail: ijehseditor@gmail.com
Website: www.stenvironment.org

Volume 2 (3): JULY - SEPTEMBER 2020 ISSN: 2582-5283

A PEER REVIEWED & REFEREED JOURNAL

International Journal of Environment and Health Sciences

EDITORIAL OFFICE
INTERNATIONAL JOURNAL OF ENVIRONMENT AND HEALTH SCIENCES

Address for correspondence: Flat No. 1107, Block 17, Heritage City, MG Road, Gurugram-122008, Haryana
Mobile: 9871 372 350 • E-mail: info@stenvironment.org

Head & Registered Office: 12, Diamond Harbour Road, Kolkata-700063
Mobile: 9871 372 350 • E-mail: info@stenvironment.org

INTERNATIONAL JOURNAL OF ENVIRONMENT AND HEALTH SCIENCES

Volume: 2 Issue: 3 JULY TO SEPTEMBER 2020

AIMS AND OBJECTIVES OF IJEHS:

The IJEHS is an official publication of Save The Environment (STE). It publishes peer reviewed quarterly, original articles (Research paper, Review articles, Short Communication, Case studies, etc.) related to all fields of Environment and Health Sciences. It disseminates the scientific research and recent innovations.

EDITORIAL BOARD

ADVISORY BOARD

INTERNATIONAL ADVISORS

Role	Name	Institution	E-mail
Advisor	**Dr Petros Koutrakis**	Professor of Environmental Sciences Harvard TH Chan School of Public Health Boston, MA, USA	petros@hsph.harvard.edu
Advisor	**Dr Satinder Ahuja**	President, Ahuja Academy of Water Quality Calabash NC, USA	sutahuja@atmc.net
Advisor	**Dr. Satinder Kaur Brar**	Professor and James and Joanne Love Chair in Environmental Engineering Department of Civil Engineering, Lassonde School of Engineering, York University CANADA	satinder.brar@lassonde.yorku.ca
Advisor	**Dr J Mikhail Wolfson**	Research Associate, Department of Environmental Sciences, Harvard TH Chan School of Public Health, Boston MA, USA	mwolfson@hsph.harvard.edu
Advisor	**Dr. Linda Schweitzer**	Emeritus Professor, Oakland University Rochester, MI & Research Scientist at Southern university A&M College in Baton Rouge Louisiana, USA	lindaschweitzer50@gmail.com
Advisor	**Prof. Amitava Adhikary**	Research Associate Professor Oakland University, Rochester MI, USA	adhikary@oakland.edu
Advisor	**Prof. Gausal A. Khan**	Professor, Department of Physiology Fiji School of Medicine Suva, Fiji	gausalk@gmail.com

ADVISORY BOARD

NATIONAL ADVISORS

Role	Name	Institution	E-mail
Advisor	**Dr. Arunabha Majumdar**	Professor Emeritus, School of Water Resources Engineering, Jadavpur University & Former Director, All India Institute of Hygiene and Public Health, Kolkata	arunabhamajumder@hotmail.com
Advisor	**Prof. RK Sharma**	Coordinator, Green Chemistry Network Centre University of Delhi, Delhi	rksharmagreenchem@hotmail.com
Advisor	**Dr. Anju Srivastava**	Principal, Hindu College, University of Delhi	dr.anjusrivastava@gmail.com
Advisor	**Dr. Shoma Paul Nandi**	Professor & Centre Head, Cellular & Molecular Bio-Technology Amity University, Noida	spaul@amity.edu
Advisor	**Dr. PK Rai**	Scientist 'G' & Joint Director CFEES, DRDO, Delhi	pramrai@rediffmail.com
Advisor	**Dr. Sanjay Bajpai**	Scientist 'G' & Head Technology Mission Division (Water and Clean Energy), DST Govt. of India, Delhi	sbajpai@nic.in
Advisor	**Dr. Susan Titus**	Scientist 'F' & Additional Director NMRL, DRDO, Ambarnath	stitus@rediffmail.com
Advisor	**Dr. Sushil K Singh**	Scientist 'F' & Additional Director SSPL, DRDO, Delhi	singhksushil@gmail.com
Advisor	**Dr. Dinesh Rangappa**	Professor & Chairman, Department of Nanotechnology VTU, Belagavi, Karnataka	dineshrangappa@gmail.com
Advisor	**Dr. Ajay Kumar Gupta**	Professor, Herbal Medicines & Natural Products Laboratory Department of Biotechnology Maharishi Markandeshwar (Deemed to be University), Ambala	akgupta.in@gmail.com
Advisor	**Prof. Sanjeev Chaudhari**	Centre for Environmental Science and Engineering, IIT Bombay, Mumbai	sanjeev@iitb.ac.in
Advisor	**Prof. Gauri Pandit**	Adjunct Professor, Centre For Environmental Science and Engineering, IIT Bombay, Mumbai	ggpandit@iitb.ac.in
Advisor	**Dr. Neelima Alam**	Scientist 'E', Technology Missions Division (Energy, Water & All Other) DST, Govt. of India, Delhi	neelima.alam@nic.in
Advisor	**Dr. C. Karunakaran**	Associate Professor, Biomedical Research lab Department of Chemistry, VHNSN College Virudhunagar, Tamil Nadu	ckaru2000@gmail.com
Advisor	**Dr. Yasmin Ahmad**	Scientist 'D', DIPAS, DRDO, Delhi	yasminchem@gmail.com
Advisor	**Dr. Nupur Bahadur**	Fellow Water Research Group, TERI-Deakin Nano Biotechnology Centre, Gurugram	nupur.bahadur@teri.res.in
Advisor	**Dr. Anuja Bhardwaj**	Research Associate, STE	anujabhardwaj75@gmail.com

International Journal of Environment and Health Sciences

From The Editor's Desk…

Advent of modern technologies is leading to persistent urbanization and capitalism which although favorable to mankind, have a plethora of environmental hazards accompany them. Air pollution, water contamination, greenhouse gas emissions, etc. are manmade tribulations that are challenging the availability of clean air and pure water. Consequently, health perils linked to environmental risk factors are snowballing at an alarming rate. In this outlook, the necessity for formation of regulatory bodies to propagate awareness for environmental sustainability is more now than ever before.

With this perspective, the International Journal of Environment and Health Sciences (IJEHS) proposes to provide a reliable platform to discuss technologies and strategies for management of aforesaid environmental matters. IJEHS has been launched as a peer-reviewed quarterly journal that will be quintessential to academicians, industry professionals and researchers who are actively engaged in the areas of environmental issues and related health effects. We are pleased to inform that ISSN for IJEHS is now available as 2582-5283. Also, IJEHS is now indexed in Crossref (DOI 10.47062) and International Scientific Indexing.

We invite original research articles, short communications and critical reviews directed towards an academic, clinical and industrial audience. The first section of the journal focuses on burning environmental issues like pollutants and their fate, waste management, resource conservation, remediation technologies, etc. The second section includes all topics relevant to physiological impact of environmental risk factors and application of alternative medicinal approaches as remedial measures. Detailed scope can be found in the home page of the journal (www.stenvironment.org). Notes on development of any novel and validated strategy or tool to address environmental challenges are welcome. Discussion on proceedings of conferences conducted around an environmental theme will also be considered. All submissions will be meticulously scrutinized by pioneers in the field to ensure publication of only articles of high quality and relevance. Authors are requested to take special precautions to avert plagiarism and redundancy.

It is high time that we realize the gravity of circumstances and take potent steps to undo the adversities already triggered. The time is now and the place is here. With this, I wish all our readers a Very Happy New Year, 2020 and I hope our audience and patrons shall come together in this effort to promulgate their part in resurrecting our valuable environment.

Dr. Kshipra Misra
Editor-in-Chief, IJEHS

International Journal of Environment and Health Sciences

Volume: 2 Issue: 3 JULY TO SEPTEMBER 2020

CONTENTS

A. Environmental Sciences Section

Sl. No.	Topic	On page
	A. Environmental Sciences Section	
1.	**ROLE OF ACID HYDROLYSIS BEHAVIOR ON STRUCTURE AND DEPOLYMERIZATION OF LIGNIN EXTRACTED FROM RICE STRAW** Sumit Sharma and Saurabh Jyoti Sarma	9-14
2.	**GROUNDWATER QUALITY ASSESSMENT FOR DRINKING PURPOSE IN SOUTH-EASTERN PART OF PANCHKULA CITY, HARYANA, INDIA** Anup Kumar, Shishupal Singh and V.S. Arya	15-21
3.	**STUDIES ON CONSORTIUM OF MARINE OIL DEGRADING BACTERIA FOR DEGRADATION OF HIGH SPEED DIESEL (HSD)** Namrata Kamble, Chitra Pawar, Rajamani Mohanram and Susan Titus	22-29
	B. Health Sciences Section	
4.	**BIOCHEMICAL CONSTITUENTS OF MEDICINAL IMPORTANT *COLEUS FORSKOHLII* BRIQ. (PATHARCHUR), UNDER SOIL-MOISTURE STRESS CONDITION** N. A. Khan, G. S. Shaktawat, S. D. Upadhyaya and A. Upadhyay	32-36
5.	**FRACTIONS OF *HIPPOPHAE RHAMNOIDES* TURKESTANICA EXTRACT AND THEIR ADAPTOGENIC AND IMMUNOMODULATORY POTENTIALS** Priyanka Sharma, Geetha Suryakumar, Himanshi Tanwar Kshipra Misra and Lilly Ganju	37-49

Annual Subscription

Individual	Rs. 5000.00
Institutional	Rs. 2000.00

Other Countries

Individual	$ 500.00
Institutional	$ 650.00

A.
Environmental Sciences Section

International Journal of Environment and Health Sciences (IJEHS)
2020, VOL. 2, NO. 3, 9–14
http://www.stenvironment.org/

ROLE OF ACID HYDROLYSIS BEHAVIOR ON STRUCTURE AND DEPOLYMERIZATION OF LIGNIN EXTRACTED FROM RICE STRAW

Sumit Sharma[1], **Saurabh Jyoti Sarma**[1*]

[1]Department of Biotechnology, Bennett University, Greater Noida, (U.P.), India

Received on: 08.06.2020 **Revised on:** 23.06.2020 **Accepted on:** 27.06.2020

Abstract
Chemical reactions mostly have a dependency on catalyst concentration. Sometimes, the action of higher concentration enhances the rate very high that disrupts the product appearance and structural integrity. It is also applicable for biological material degradation. Here in this study, the degradation of rice straw biomass was executed for lignin extraction using the acid hydrolysis treatment method. This technique helps in the removal of maximum cellulosic parts like cellulose and hemicellulose by converting them into soluble sugars and remains left out lignin. It was investigated that the higher concentration of sulfuric acid as 72% v/v (80.77% w/w) caused some structural changes in chemical bonds and formed highly condensed lignin (L-72). While mild concentration of sulfuric acid 63% v/v (72.26% w/w) does not have any adverse effect on lignin structural integrity and was found as free-form lignin (L-63). The impact of condensation was observed during the depolymerization of L-72 and L-63. The depolymerization efficiency of L-72 and L-63 in alkaline medium (NaOH 1.5% and Na2S 0.5%) comparatively lesser for L-72 (34%) than L-63 (98.3%) using lignin 2 g/l.FT-IR analysis also showed the presence of CO-O-CO (anhydride) and C=C (alkenes) in condensed lignin but not found in free-form lignin. This means the structural condensation decreasing the depolymerization efficiency of lignin. Hence, it is concluded that free-form, light- brown lignin should be used for depolymerization and monolignolextraction.

Keywords
Acid hydrolysis, Condensed Lignin, Free-form lignin, Depolymerization

Introduction

Lignin waste management and depolymerization is the major challenge facing by cellulosic refinery and wood pulp industries [1, 2]. Solid remaining lignin is becoming the major waste there. Lignin utilization as a simple source is difficult due to its polymeric structure that is made up of aromatic rings [3]. These rings are divided into 3 kinds of aromatic alcohols (sinapyl, coniferyl, and coumaryl forms) bound by aryl-ether bonds. These bonds are not easily breakable by the acid treatment process used recently in biorefineries to obtain higher cellulosic sugar yield [4]. The lignin generated from these treatments is a phenolic complex [5] and its depolymerization depends on structural integrity. The depolymerization rate is directly linked with the extent of lignin complexity. If it is too high, the bond breakage is very difficult and needs a higher temperature with a catalyst [6]. More complex means condensed lignin and less complex simpler form can be called free-form lignin. For complete hydrolysis of sugar, a higher concentration of acid can be used but it may de-hydrate the other reactive compound and condensed them into the structure. This causes difficulty in further degradation of lignin into a product. The use of this abundant waste requires a simpler process for its treatment such as depolymerization.Alkaline-based depolymerization is a way to convert into monomers [7]. While due to high complexity, the access of alkaline functional components such as –OH and –SH group to oxidize the lignin into simple monomers become unavailable and the free space, as well as action site, closed inside the complex [8]. The nucleophilic attack of the functional group of solvent is only accessible to

CONTACT *Corresponding author: saurabh.sarma@bennett.edu.in
Color versions of one or more of the figures in this article can be found online at www.stenvironment.org

the outer surface and the inter-bound aryl-ether bonds become inaccessible [8, 9]. This directly affects the decrease in the rate of depolymerization of lignin. Here in this study, the effect of acid concentration on lignin extraction from rice straw biomass and its depolymerization challenges are focused. It is also attempted to find out how structural complexity plays role in lignin de-polymerization efficiency.

MATERIALS AND METHODS

Dry biomass of rice straw was collected from the region of Mohna, Ballabgarh, Faridabad, Haryana. All the chemicals used in this study have been procured from SRL chem.This study used the methodsexplained in further sub-sectionsfor identifying the role of acid hydrolysis on the lignin extraction, depolymerization.Along with that, substrate value optimization and structural analysis has also been done for validation of the comparative outcomes.

Condensed and free-form lignin extraction from rice straw

Rice straw lignin was extracted by usinga method developed by Sluiter et al. (2008) [10] along with two different concentrations of sulfuric acid 80% w/w and 72% w/w. The method was followed as: rice straw was subjected to acid solutions in a 1:10 ratio and kept for incubation for 60 minutes at 30°C at 150 RPM. After incubation, diluted to 4 % by calculating the dilution in v/v forms: 72% v/v =80.77% w/w) and 63% v/v =72.26% w/w. Both the mixtures were autoclaved at 121°C for 30 minutes at 15 psi. After cool down, the solid was separated from the liquid by centrifugation at 8000 rpm for 10 minutes at 25°C. The solid pellet was washed 2 times with distilled water (DW) and then dried at room temperature (RT) for 24-48 hours for complete drying of the solid. The solid material was weighed to find out lignin percentage and then stored in glass vials at room temperature for further use. Different parameters like the appearance of color; texture and solubility in water were investigated.

Depolymerization of condensed and free-form lignin

De-polymerization of both types of lignin was done with an alkaline solution of NaOH (1.5%) and Na_2S (0.5%). This combination of **S**olution for **C**hemical-bond **B**reakagewas named SCB. 2g/l of L-72 and L-63 were mixed with SCB in two sets of temperatures:30°C and 80°C. Both the combinations were incubated at both temperatures (in triplicates) for 60 minutes, mixed after every 10 minutes. After incubation, removed from respective specific temperatures and kept for settling at RT for 1 hour. The liquid was removed by pipetting and subjected to neutralization by 1M H_2SO_4 to nearly pH 7-8. and the solid was washed 2 times with DW. The Solid-water mixture was washed and separated bycentrifugation at 8000 RPM for 10 minutes at RT and dried at RT for 12 hours and dried remaining lignin was weighed for calculating depolymerization efficiency.

Substrate concentration optimization near to maximum rate

The substrate concentration optimization was done with L-63 ranges from 1g/l to 5g/l in duplicates with SCB. The experiment was done in 30 ml screw-cap glass vials with 10 ml working volume. The mixture was incubated at 30°C for 60 minutes at 150 RPM.A similar experiment was done with L-63 from 0.5 g/l to 2.5 g/l values at 30°C for identifying the closest substrate value. After incubation, depolymerization efficiency was estimated by a similar procedure applied before.

FT-IR analysis for the structural integrity of L-72 and L-63

Fourier transform infrared (FT-IR) spectrum analysis was done for identifying the carbon bond stretching and bending which represents the structural complexity of a compound. L-72 and L-63 extracted fine powder samples were placed on the sample platform on the FT-IR spectrometer (PerkinElmer Spectrum Two™). The sample was scanned using the software Spectrum10 at 400 to 4000 wave number (cm^{-1}) for estimating transmittance. Firstly, a background blank was run to normalized all the background noise. Then, samples were run for extracting transmittance in percentage with respect to wave number from 400 to 4000 cm^{-1}. The comparison profile of inter-linkage bonds was analyzed after plotting a graph between wave number (cm^{-1}) vs transmittance (%).

RESULTS AND DISCUSSION

Condensed vs Free-form lignin characteristics

The condensed and free-form lignin extracted with two different concentrations of sulfuric acid showed a great impact on their product formation. The condensed lignin formed by 80% sulfuric acid (w/w) was dark brown in appearance while free-form formed by 72% sulfuric acid (w/w) was light brown. A high dense weight L-72 and lightweight L-63indicated that as the concentration of acid increases the condensation of lignin increases and structure became more complex and denser. Xu et al. (2006), also found an increase in condensation of lignin with an increase of organic acid in wheat straw pre-treatment [11] and the reason behind this was an increase of guaiacol units in biomass. It was also proposed by Sannigrahi et al. (2011) that condensation at higher acid leads to the production of pseudo-lignin [12] and enhances the complexity. At higher acid catalysis, dehydration of sugars generates furans, and these furans ties with aromatic precursors of lignin resulted in highly condensed polymeric lignin [4]. L-72was observed asa highly condensed form of lignin. L-72 and L-63 comparison at different parameters is illustrated in Table 1. The texture was observed asrough and hard for L-72 whereas smooth and soft for L-63. When solubility was tested in water, it was found that both are insoluble in distilled water.

The final weightof both the lignin was nearly similar to 30-32% ofthe total dry weight of rice straw biomass, so there was a negligible difference in loss of solid lignin during acid treatment (Table 1). But the condensation of lignin was observed at a higher acid concentration of 80% w/w and free-form lignin at a slightly low concentration of 72% w/w. Figure 1 depicted the visible appearance of both types of lignin. The surface area on visual appearance was also observed lowerfor condensed lignin than free-form lignin when a similar amount of lignin was valuedin mass. After all the comparative analysis, it was found that the L-63 is actual lignin that can be used further for depolymerization and monolignol extraction, while the comparative depolymerization study was done with both kinds of lignin to validate their structural complexity.

Figure 1: Condensed (L-72) and free-form (L-63) lignin extracted from rice straw by acid hydrolysis.

Table 1: Comparison between L-72 and L-63 at different parameters.

Sr. No.	Different Parameters	L-72	L-63
1	Color	Dark brown	Light brown
2	Texture	Rough	Smooth
3	Density	High	Low
4	Solubility in water	Insoluble	Insoluble
5	Lignin (%)	31±1	31±1
6	Visible Surface area	Small	Large

The rate of depolymerization of both the lignin was identified by treating with previously optimized alkali SCB: Sodium hydroxide (NaOH): 1.5% and Sodium sulfide ($Na_2S.xH_2O$) 0.5% – treatment based process for lignin (data not shown). The 2g/l of L-72 treated with SCB showed very less depolymerization at both the temperature 30°C and 80°C as compared to L-63 in both the conditions. A step-wise schematic representation for the process was also given in Figure 2. The dense form of L-72 does not show proper dissolution in an alkaline medium while the free-form L-63is easily dissolved in it. It was found that L-63 de-polymerized 88.3% at 30°C and 98.3% at 80°C, while L-72 de-polymerized 30% at 30°C and 34% at 80°C (Figure 3). The effect of pH neutralization was observed by the formation of precipitate while lowering the pH to near 7 (Figure 4). The L-63 at 30°C showed no precipitation after neutralization while a white precipitate was formed at near pH 7-8 in other samples. This showed that the L-63 does not show coagulation of free monolignols into precipitated lignin when pH reached near neutralization. Generally, reaction with acid causes phenolic group protonation [13] leads to the coagulation and precipitation of free monolignol of lignin. The acid precipitation process is used in paper industries for lignin recovery[14]. Mostly, lignin recovery from industry pulp liquor was done by reducing pH lower than 7 by the addition of acid [15]. In this case, ML (Monolignols; shown in Figure 4) of L-63 at 30°C is fairly stable at near neutral pH & and can be further proceeded to separate monomer purifications.

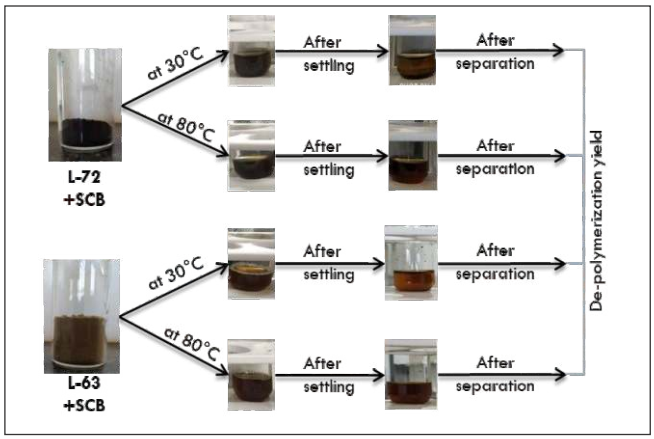


Figure 2: Step of L-72 and L-63 de-polymerization at two different temperature with 2g/l of lignin in SCB.

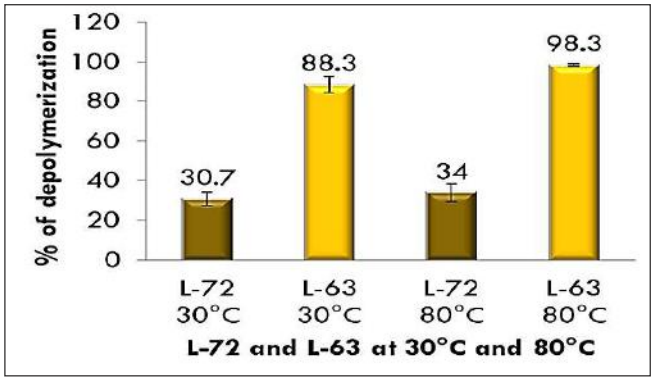


Figure 3: L-72 and L-63 depolymerization efficiency estimations at 30°C and 80°C.

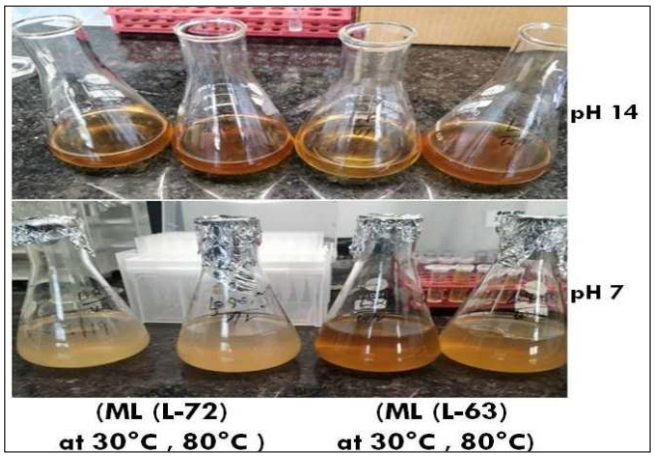


Figure 4: Effect of pH on L-72 and L-63.

Substrate optimization of higher range L-63

It was verified from the above experiment that the L-63 is better than L-72. Then, the maximum L-63 depolymerization with SCB was optimized at the higher range of L-63 concentration from 1g/l to 5g/l. After depolymerization reaction, it was found that the 1g/l and 2 g/l of L-63 depolymerized completely near 85% at 30°C, but when concentration increased 3g/l to 5 g/l depolymerization efficiency was <70 % (data not shown). So that further experiment was done to find out near maximum value and reproducibility of L-63. The range from 0.5g/l to 2.5 g/l of L-63 was evaluated for depolymerization efficiency using SCB. It was found that all the values have more than 80% depolymerization efficiency at temperature 30°C (Figure 5). It concludes that a higher substrate value of 2g/l of L-63can be used for depolymerization into monolignols and further purification of different phenolic monomers.

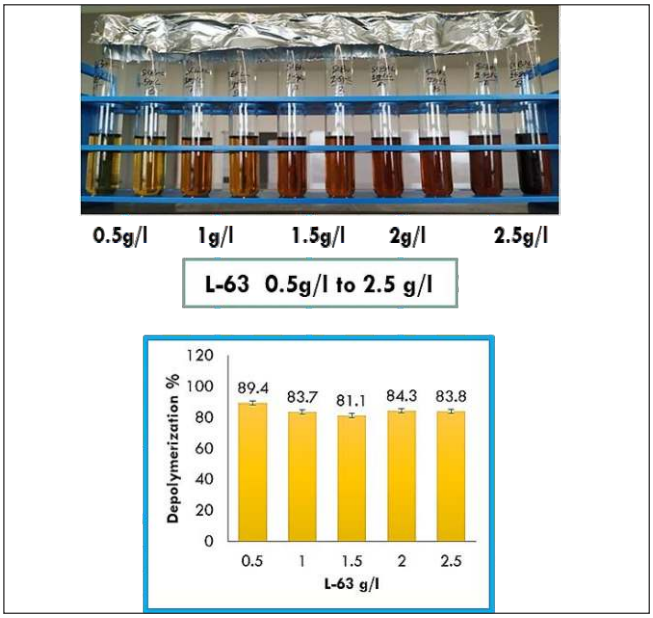


Figure 5: Substrate optimization for L-63 at different concentration of lignin in SCB.

FT-IR interlinkages in L-72 and L-63

The FT-IR plot curve depicts the significant difference in the interlinking bonds between the L-72 and L-63 structural characteristics. It was found that the L-72 lignin sample consists of CO-O-CO (anhydride) and C=C (alkene) stretching and more carbon-carbon bond [16]showed the lignin interlinkages. While, in L-63, there were simple C-O and C=Ostretching were found which showed the free form of the lignin structure (Figure 6). Remil et. al (2014) also found that the position of lignin falls into a similar range of wave number between 1700-1300 cm^{-1}as the maximum number of carbon bond stretching are found in this study at same

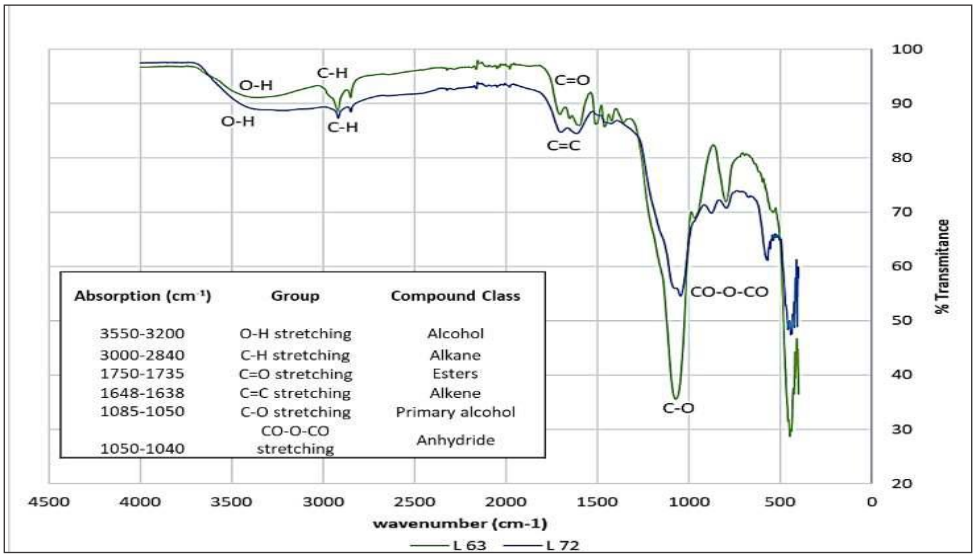


Figure 6: FT-IR spectrum showing the bond interlinkage comparison of the structural changes in lignin L-72 and L-63.

Proposed structural integrity of the L-72 and L-63

The depolymerization rate is directly linked with the extent of lignin complexity. If it is too high, the bond breakage is very difficult, it dehydrates the reactive compound and condensed them more and more. This causes difficulty in further degradation of them into other products. Due to high complexity, the access of alkaline reagents becomes inaccessible and the free space and action site closes inside the complex. Guadix-Montero [19] and Shuai [20] explained the presence of inaccessible and unbreakable bonds formed due to condensation of reactive compounds in the lignin that occurred during the extraction process. A diagrammatic representation is proposed in Figure 7 to differentiate the structural integrity of the condensed and free-form lignin based on bond linkages between different precursors of lignin. There are chances of more number of unbreakable bond and inaccessible bond are present in the L-72, while a lesser number in L-63. The breakable bond can be present inside the vicinity of complex which are more accessible in L-63 than L-72 because L-63 is more accessible from the surface also. Therefore, free-form lignin (L-63) showed higher depolymerization efficiency than condensed lignin (L-72).

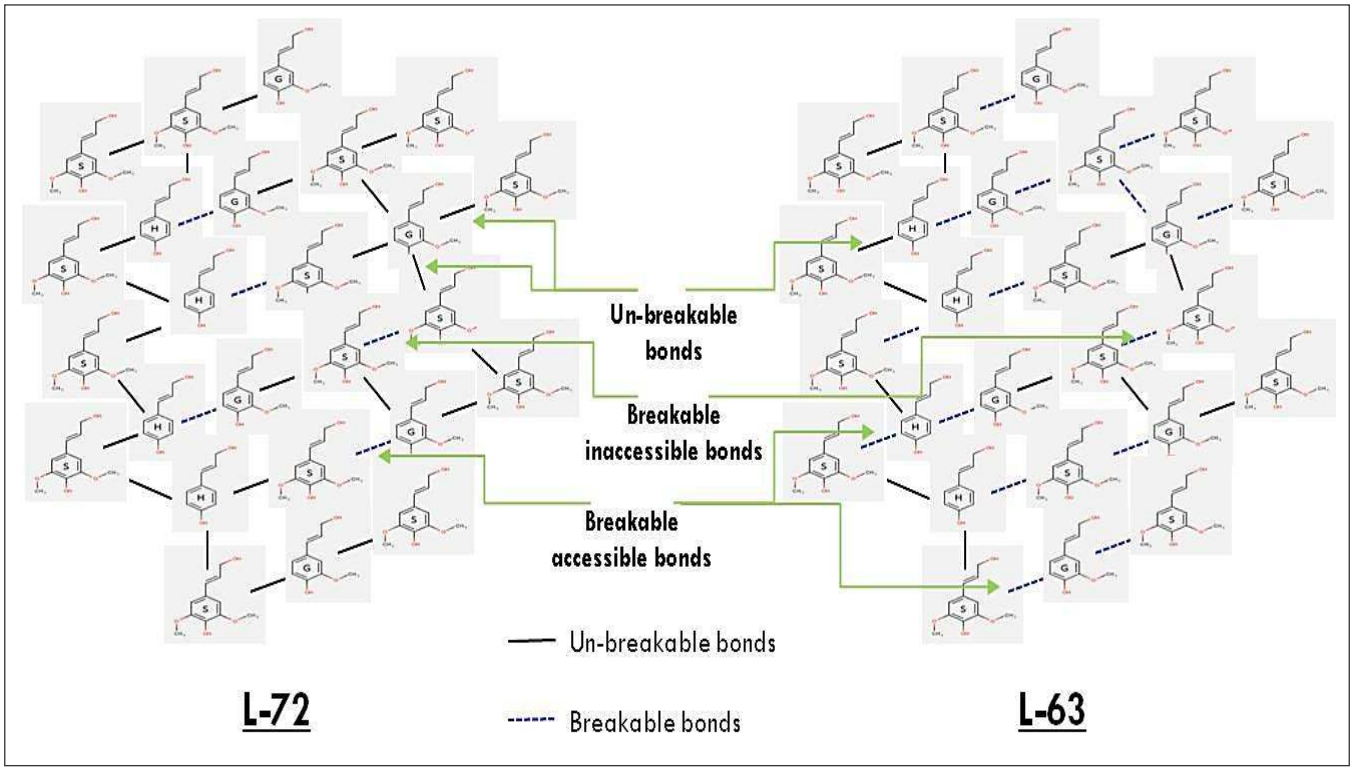


Figure 7: Proposed diagram of the structural integrity of the L-72 and L-63: Occurrence of inaccessible, breakable, and unbreakable bonds.

CONCLUSION

The selection of a better lignin substrate is the major key-base for de-polymerization studies. The effect of acid concentration change was found to directly affect the lignin structural changes. Similarly, further depolymerization efficiency was also affected. The availability of a free form of lignin groups is needed for the action of reactive functional groups of reagents. The use of the alkaline medium was very effective in depolymerizing nearly the maximum amount of L-63 and only one-third forL-72. So, it was concluded to use the free-form, soft, and light- brown lignin (L-63) for depolymerization and monolignol extraction. It also helps inthe purification and separation of phenolic monomers.

ACKNOWLEDGEMENTS

The authors are thankful to Bennett University for providing the necessary financial assistance to complete this study.

CONFLICT OF INTEREST

The authors declared no conflict of interest in this study.

REFERENCES

1. **Li T, Takkellapati S.** The current and emerging sources of technical lignins and their applications. Biofuels, Bioproducts and Biorefining. 2018 Sep;12(5):756-87.
2. **Bruijnincx PC, Rinaldi R, Weckhuysen BM.** Unlocking the potential of a sleeping giant: lignins as

sustainable raw materials for renewable fuels, chemicals and materials. Green Chemistry. 2015;17(11):4860-1.

3. https://edu.rsc.org/feature/the-lignin-challenge/2000124.article (accessed on date 16/11/2020)

4. **Yoon SY, Han SH, Shin SJ.** The effect of hemicelluloses and lignin on acid hydrolysis of cellulose. *Energy*. 2014 Dec 1;77:19-24.

5. **Sannigrahi P, Ragauskas AJ, Miller SJ.** Effects of two-stage dilute acid pretreatment on the structure and composition of lignin and cellulose in loblolly pine. BioEnergy Research. 2008 Dec 1;1(3-4):205-14.

6. **Moxley G, Gaspar AR, Higgins D, Xu H.** Structural changes of corn stover lignin during acid pretreatment. Journal of industrial microbiology & biotechnology. 2012 Sep 1;39(9):1289-99.

7. **Katahira R, Mittal A, McKinney K, Chen X, Tucker MP, Johnson DK, Beckham GT.** Base-catalyzed depolymerization of biorefinerylignins. ACS Sustainable Chemistry & Engineering. 2016 Mar 7;4(3):1474-86.

8. **Shuai L, Saha B.** Towards high-yield lignin monomer production. Green Chemistry. 2017;19(16):3752-8.

9. **Svensson S.** Minimizing the sulphur content in Kraft lignin. 2008. http://www.diva-portal.org/smash/get/diva2:1676/FULLTEXT01.pdf (accessed on date 29/11/2020).

10. **Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker DL.** Determination of structural carbohydrates and lignin in biomass. Laboratory analytical procedure. 2008 Apr 25;1617(1):1-6.

11. **Xu F, Sun JX, Sun R, Fowler P, Baird MS.** Comparative study of organosolvlignins from wheat straw. Industrial crops and products. 2006 Mar 1;23(2):180-93.

12. **Sannigrahi P, Kim DH, Jung S, Ragauskas A.** Pseudo-lignin and pretreatment chemistry. Energy & Environmental Science. 2011;4(4):1306-10.

13. **Zhu W, Theliander H.** Precipitation of lignin from softwood black liquor: an investigation of the equilibrium and molecular properties of lignin. BioResources. 2015 Jan 29;10(1):1696-714.

14. **Lourençon TV, Hansel FA, da Silva TA, Ramos LP, de Muniz GI, Magalhães WL.** Hardwood and softwood kraftlignins fractionation by simple sequential acid precipitation. Separation and Purification Technology. 2015 Nov 5;154:82-8.

15. **Domínguez-Robles J, Espinosa E, Savy D, Rosal A, Rodríguez Pascual A.** Biorefinery process combining specel® process and selective lignin precipitation using mineral acids.

16. https://www.sigmaaldrich.com/technical-documents/articles/biology/ir-spectrum-table.html (Accessed on date 10/12/2020).

17. **Remli NA, Shah UK, Mohamad R, Abd-Aziz S.** Effects of chemical and thermal pretreatments on the enzymatic saccharification of rice straw for sugars production. BioResources. 2014;9(1):510-22.

18. **Watkins D, Nuruddin M, Hosur M, Tcherbi-Narteh A, Jeelani S.** Extraction and characterization of lignin from different biomass resources. Journal of Materials Research and Technology. 2015 Jan 1;4(1):26-32.

19. **Kong F, Parhiala K, Wang S, Fatehi P.** Preparation of cationic softwood kraft lignin and its application in dye removal. European Polymer Journal. 2015 Jun 1;67:335-45.

20. **Guadix-Montero S, Sankar M.** Review on catalytic cleavage of C–C inter-unit linkages in lignin model compounds: towards lignin depolymerisation. Topics in Catalysis. 2018 Apr 1;61(3-4):183-98.

International Journal of Environment and Health Sciences (IJEHS)
2020, VOL. 2, NO. 3, 15–21
http://www.stenvironment.org/

GROUNDWATER QUALITY ASSESSMENT FOR DRINKING PURPOSE IN SOUTH-EASTERN PART OF PANCHKULA CITY, HARYANA, INDIA

Anup Kumar[1*], Shishupal Singh[2] and V.S. Arya[3]

[1]Front Office-HARSAC, Panchkula, India
[2]Govt. ITI, Panchkula, India
[3]Haryana Space Applications Centre (HARSAC), Hisar, India

Received on: 01.07.2020 **Revised on:** 21.07.2020 **Accepted on:** 25.07.2020

Abstract
Water is a precious natural resource available on the planet Earth. About two third is water on the planet Earth but the usable water mainly for drinking purpose is very less. Fast developmental activities have put pressure on surface water and groundwater. Groundwater is highly important for drinking, agriculture and industrial purposes in the world. Groundwater quality plays an important role for drinking purpose. Many diseases like fluorosis, methemoglobinemia, arsenocosis are due to poor quality of drinking water. In urban areas, groundwater quality is deteriorated due to solid and liquid wastes disposal. The present study has been carried out to assess groundwater quality for drinking purpose in south-eastern part of Panchkula city, Haryana. In the present study eight groundwater samples were collected in the month of June 2019 from different locations in the study area. Groundwater samples were analyzed using Field Water Testing Kit prepared by Tamilnadu Water Supply and Drainage Board (TWAD), Chennai for ten chemical parameters-pH,hardness, chloride, fluoride, iron, ammonia, nitrate, nitrite, phosphate and residual chlorine. Chemical analysis of groundwater samples show that in the groundwater samples pH ranges-7 to 7.5, hardness 100 mg/l to 250 mg/l, chloride 40 mg/l to 110 mg/l, fluoride 0.5 mg/l to 1 mg/l, iron nil, ammonia nil to 3 mg/l, nitrite 0.2 mg/l to 0.5 mg/l, nitrate 20 mg/l to 75 mg/l, phosphate nil to 1 mg/l, residual chlorine nil to 0.2 mg/l. As per BIS drinking water standards pH, chloride, fluoride, iron, nitrite, phosphate, residual chlorine is desirable in all the eight groundwater samples; hardness is desirable in four groundwater samples and permissible in four groundwater samples; ammonia is desirable in six groundwater samples and non-potable in two groundwater samples; nitrate is desirable in seven groundwater samples and non-potable in one groundwater sample. Groundwater quality at Govt. Primary School, Ramgarh, Govt. Senior Secondary School, Ramgarh and Sector-28-C, Panchkula is desirable and at Market Ramgarh, Sector-25, Panchkula, Sector-28-A, Panchkula, Sector-28-B, Panchkula is permissible and Sector-26, Panchkula is non-potable. The study gives a scenario of groundwater quality for drinking purpose in the study area. The study can be used for monitoring groundwater quality for drinking purpose.

Keywords
Groundwater, quality, assessment, drinking, Ramgarh, Panchkula, Haryana

Introduction

Water is important for survival of living beings. In the present developmental activities water resources are under stress due to utilization for drinking, irrigation and industrial purposes. Groundwater is vulnerable to anthropogenic activities wherever it is shallow and exploitation wherever there is high population pressure. In the present scenario it becomes necessary to have check on the industries polluting the groundwater as well as wise use of water in each sector. Further, in urban and semi-urban areas groundwater is under

CONTACT *Corresponding author: anup0106@yahoo.com
Color versions of one or more of the figures in this article can be found online at www.stenvironment.org

severe exploitation for drinking and industrial purposes which lead to pollution and decline of groundwater level.Abbulu and Srinivasa Rao (2013), Agrawal (2009), Deshpande and Aher (2012), Madhav et al. (2018), Tripathi etal. (2012), Zidi et al. (2017) had done work on groundwater quality assessment of urban areas. Here, the main objective of the study was to assess groundwater quality for drinking purpose in the study areas as discussed below.

Study area

The study area comprises of south-eastern part of Panchkula city (Sector 25, Sector-26, 27, 28, 29, 30 of Panchkula cityand Ramgarh town).The geo-coordinates of the study area are latitude30.64°N to 30.68°N and longitude 76.87°E to 76.89° E and covers 32.49 Km^2 area (Fig.1).

Collection of samples and analysis

Eight groundwater samples were collected in plastic 250 ml bottles during June 2019. Location of groundwater samples were marked using mobile GPS. All the eight groundwater samples were analyzed using Field Water Testing Kit prepared by Tamil Nadu Water Supply and Drainage Board (TWAD), Chennai for pH, hardness, chloride, fluoride, iron, nitrite, nitrate, ammonia, phosphate and residual chlorine. The results of chemical analysis of groundwater samples were put in MS Excel and bar graphs of each chemical parameter were prepared. Results were interpreted in comparison with BIS (IS 10500:2012) drinking water standards.

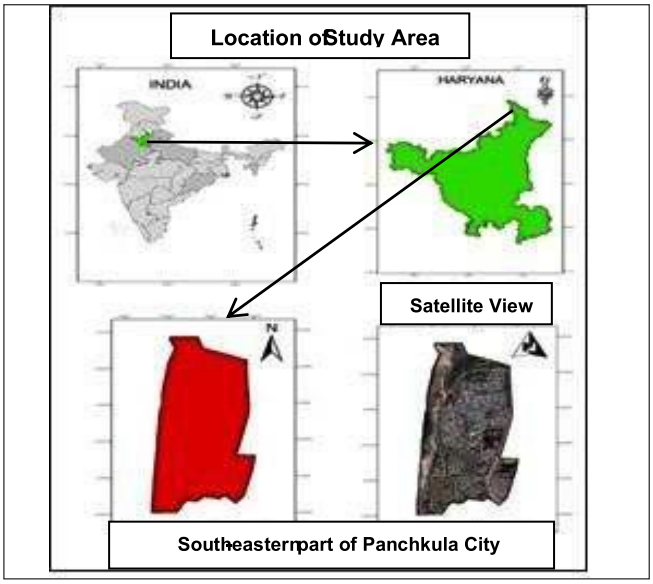


Figure 1: **Location map of the study area.**

Table 1: Groundwater samples location.

S. No.	Location	Source	Latitude	Longitude
1	Govt. Primary School, Ramgarh	Tube Well	30°39'2.65"N	76°53'20.04"E
2	Govt. Senior Secondary School, Ramgarh	Tube Well	30°38'48.85"N	76°53'5.54"E
3	Market, Ramgarh	Tube Well	30°38'44.20"N	76°53'3.10"E
4	Sector 28-A, Panchkula	Tube Well	30°38'47.89"N	76°52'57.88"E
5	Sector 28-B, Panchkula	Tube Well	30°38'52.81"N	76°52'53.38"E
6	Sector-28-C, Panchkula	Hand pump	30°38'48.35"N	76°52'33.65"E
7	Sector-26, Panchkula	Tube Well	30°39'27.41"N	76°52'51.02"E
8	Sector-25, Panchkula	Tube Well	30°40'7.40"N	76°52'38.96"E

Table 2: Results of groundwater samples analysis.

S. No.	Sample Locations	Source	pH	Hardness (mg/l)	Chloride (mg/l)	Fluoride (mg/l)	Iron (mg/l)	Ammonia (mg/l)	Nitrite (mg/l)	Nitrate (mg/l)	Phosphate (mg/l)	Residual Chlorine (mg/l)
1	Govt. Primary School, Ramgarh	Tubewell	7.5	180	40	0.5	0.0	0.5	0.5	45	0.5	0.0
2	Govt. Senior Secondary School, Ramgarh	Tubewell	7.5	100	40	0.5	0.0	0.0	0.5	45	0.0	0.0
3	Sector-28-A, Panchkula	Tubewell	7.0	250	40	0.0	0.0	0.5	0.2	45	0.5	0.0
4	Sector-28-B, Panchkula	Tubewell	7.0	250	40	0.0	0.0	0.5	0.2	45	0.5	0.0
5	Sector-28-C, Panchkula	Handpump	7.5	200	110	1.0	0.0	3.0	0.2	20	0.5	0.2

6	Sector-26, Panchkula	Tubewell	7.5	140	40	0.5	0.0	1.0	0.2	75	1.0	0.0
7	Sector-25, Panchkula	Tubewell	7.0	240	40	0.0	0.0	0.5	0.5	45	0.0	0.0
8	Market, Ramgarh	Tubewell	7.0	250	40	0.5	0.0	0.0	0.5	45	1.0	0.0

Note: A,B,C represent the three sample locations in Sector-28,Panchkula, not the Sector-28A,28B and 28C.

Table 3: BIS Drinking Water Standards (IS 10500:2012)

S. No.	Constituent	Potable		Non-Potable
		Desirable	Permissible	
1	pH	6.5 to 8.5	-	<6.5 to >8.5
2	Total Hardness (mg/l)	<200	200-600	>600
3	Chloride (mg/l)	<250	250-1000	>1000
4	Fluoride (mg/l)	<1.0	1.0-1.5	>1.5
5	Iron (mg/l)	<0.3	-	>0.3
6	Ammonia (mg/l)	<0.5	-	>0.5
7	Nitrite (mg/l)	<1.0	-	>1.0
8	Nitrate (mg/l)	<45	-	>45
9	Phosphate (mg/l)	<1.0	-	>1.0
10	Residual Chlorine (mg/l)	<0.2	0.2-1	>1.0

RESULTS AND DISCUSSION

pH

In the study area, pH varied from 7 to 7.5 and desirable in all eight groundwater samples (Fig.2).

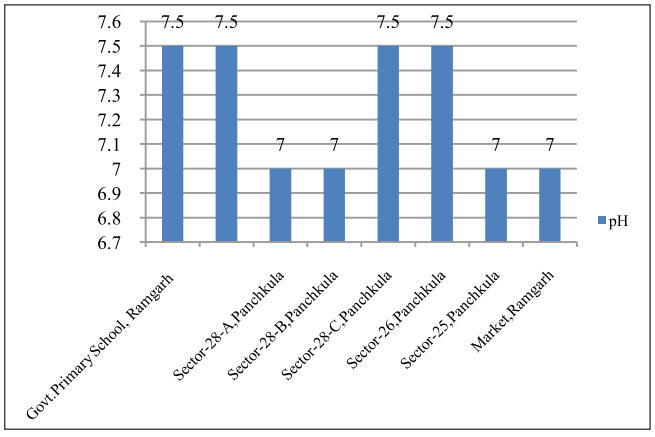


Figure 2:pH in groundwater samples.

Hardness

Hardness varied from 100 mg/l to 250 mg/l in the study area. Hardness in groundwater samples was desirable at Govt. Primary School Ramgarh (180 mg/l), Govt. Senior Secondary School Ramgarh (100 mg/l), Sector-28-C, Panchkula (200 mg/l), Sector-26, Panchkula (140 mg/l) and permissible at Sector-28-A, Panchkula (250 mg/l), Sector-28-B, Panchkula (250 mg/l), Sector- 25 (240 mg/l) and Market Ramgarh (250 mg/l) (Fig.3).

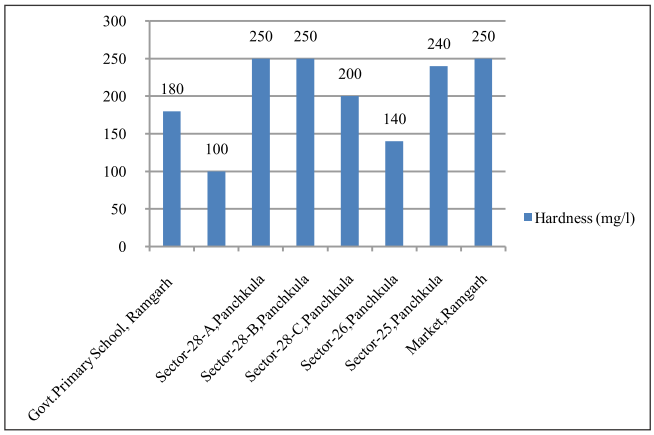


Figure 3: Hardness in groundwater samples.

Chloride

Chloride was present in range from 40 mg/l to 110 mg/l in the study area. Chloride in groundwater samples was within desired limits in all eight groundwater samples (Fig. 4).

Fluoride

Fluoride had values from 0.5mg/l to 1mg/l in the study area, thus being within desirable limits in all eight groundwater samples (Fig. 5).

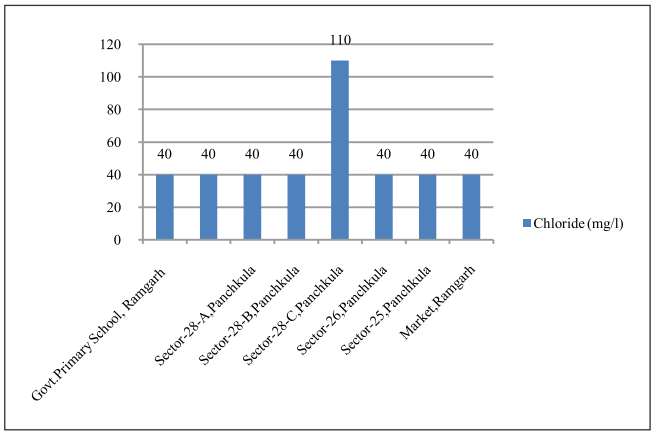


Figure 4: Chloride in groundwater samples.

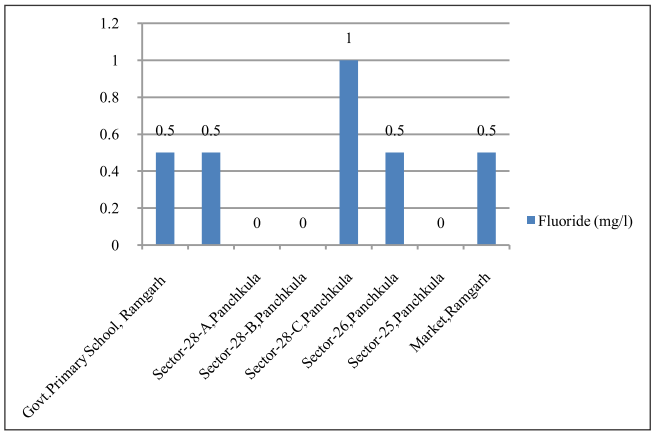


Figure 5: Fluoride in groundwater samples.

Iron

Iron was absent in all eight groundwater samples, hence, desirable for drinking purpose (Fig.6).

Ammonia

Ammonia varied over nil to 3 mg/l in the study area. Ammonia was in prescribed values atGovt. Primary School, Ramgarh (0.5 mg/l), Govt. Senior Secondary School Ramgarh (0.0 mg/l), Sector- 28-A, Panchkula (0.5 mg/l), Sector- 28-B, Panchkula (0.5 mg/l), Sector- 25, Panchkula (0.5 mg/l), Market Ramgarh (0.0 mg/l) and non-potable at Sector-28-C, Panchkula (3.0 mg/l) and Sector-26, Panchkula (1 mg/l) (Fig.7).

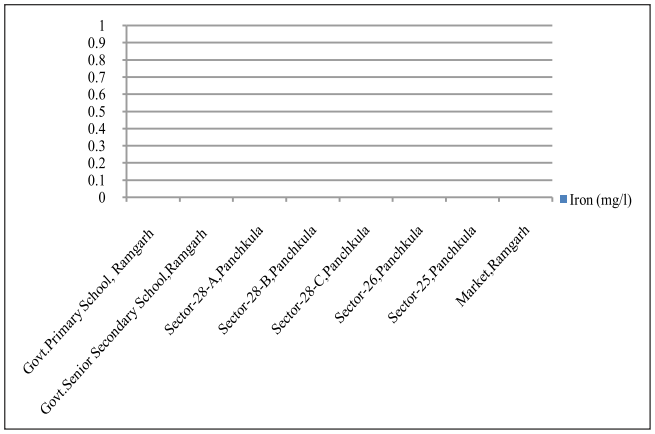


Figure 6: Iron in groundwater samples.

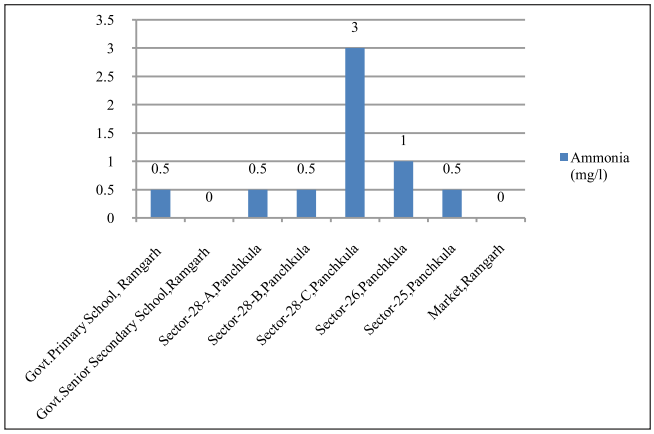


Figure 7: Ammonia in groundwater samples.

Nitrite

Nitrite varied from 0.2 mg/l to 0.5 mg/l in the study area, desirable for drinking purpose in all eight groundwater samples (Fig.8).

Nitrate

Nitrate varies from 20 mg/l to 75 mg/l in the study area, desirable in seven groundwater samples and non-potable in Sector-26 (75 mg/l) groundwater sample (Fig.9).

Phosphate

Phosphate was present over nil to 1mg/l in the study area, thus at desirable values in all eight groundwater samples (Fig.10).

Residual Chlorine

Residual Chlorine had values from nil to 0.2 mg/l in the study area (desirable) (Fig.11).

Groundwater quality at sample sites

Groundwater Quality at Govt. Primary School, Ramgarh

In groundwater sample collected at Govt. Primary School, Ramgarh, the various chemical drinking water parameters studied were found to be withindesirable limits (Fig.12).

Groundwater quality at Govt. Senior Secondary School, Ramgarh

In groundwater sample collected at Govt. Senior Secondary School, Ramgarh chemical drinking water parameters were in desirable limits (Fig.13).

Groundwater quality at Market, Ramgarh

In groundwater sample collected at Market Ramgarh analyzed chemical drinking water parameters pH, chloride, fluoride, iron, ammonia, nitrite, nitrate, phosphate, residual

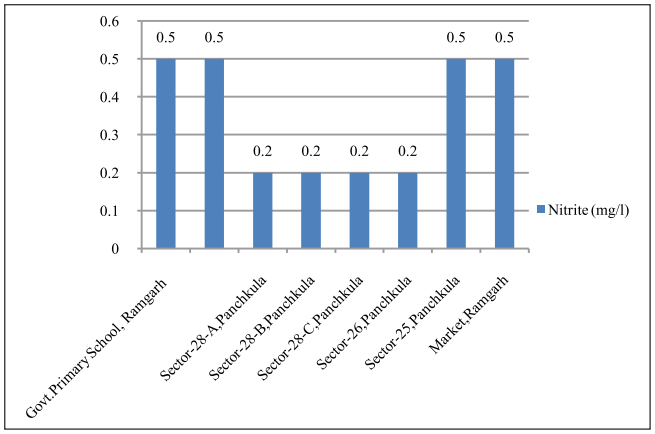


Figure 8: Nitrite in groundwater samples.

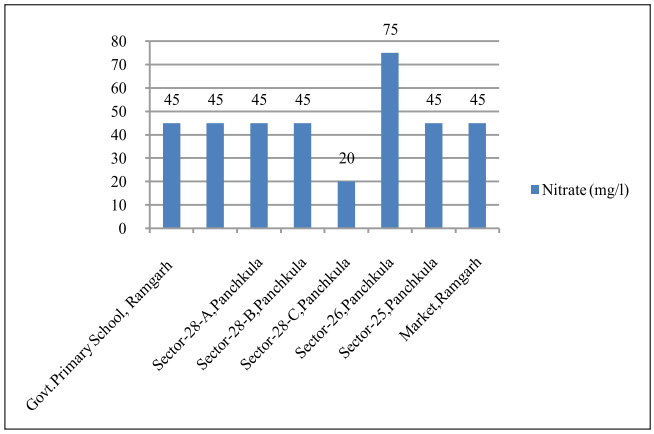


Figure 9: Nitrate in groundwater samples.

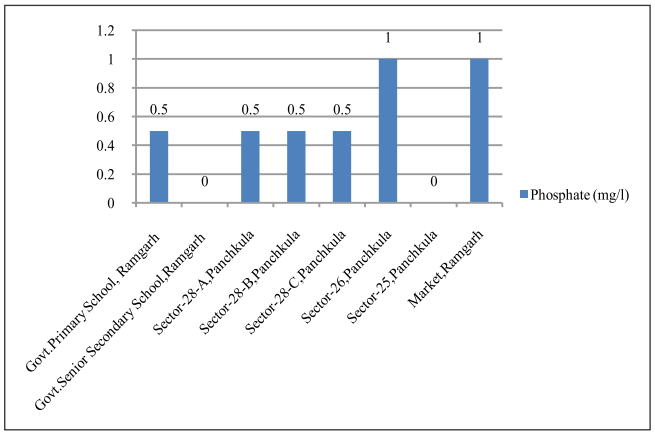


Figure 10: Phosphate in groundwater samples.

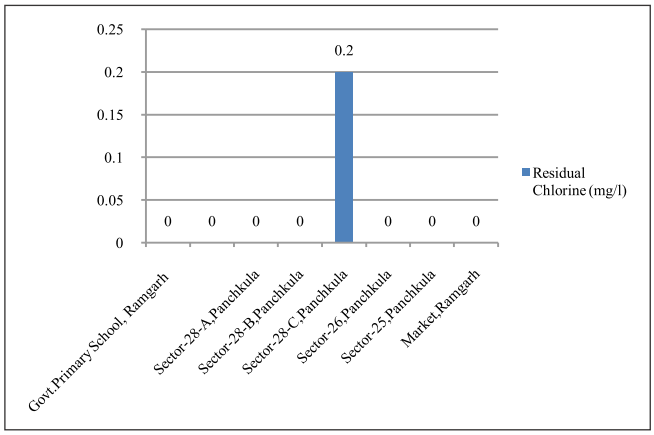


Figure 11: Residual Chlorine in groundwater samples.

chlorine were in values as permissible for drinking water (Fig.14).

Groundwater quality at Sector-25, Panchkula

In groundwater sample collected at Sector-25, Panchkula the pH, chloride, fluoride, iron, nitrite, ammonia, nitrate, phosphate, residual chlorine parameters adhered to permissible drinking water limits (Fig.15).

Groundwater quality at Sector-26, Panchkula

In groundwater sample collected at Sector-26, Panchkula the chemical drinking water parameters were under limited to desirable values and ammonia, nitrate were proper for non-potable drinking water (Fig.16).

Groundwater quality at Sector-28-A, Panchkula

In groundwater sample collected at Sector-28-A, Panchkula the different drinking water parameters were found to be

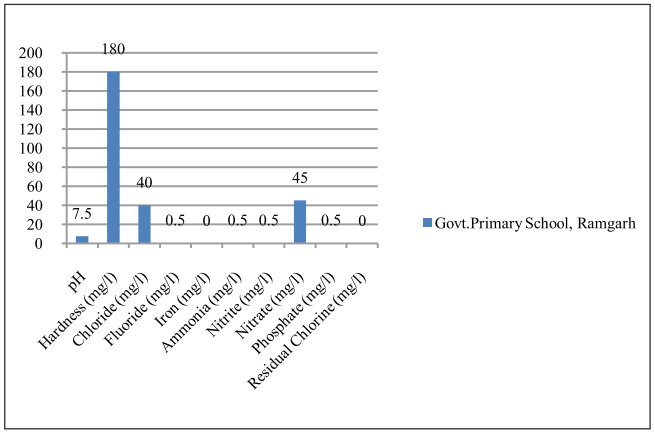


Figure 12:Groundwater quality at Govt. Primary School, Ramgarh.

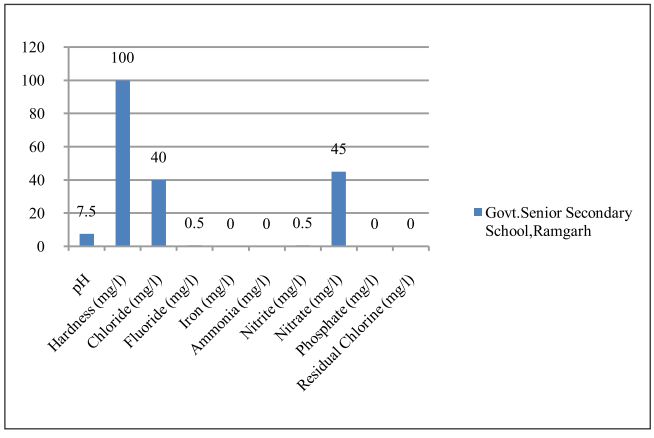


Fig.13: Groundwater quality at Govt. Senior Secondary School, Ramgarh

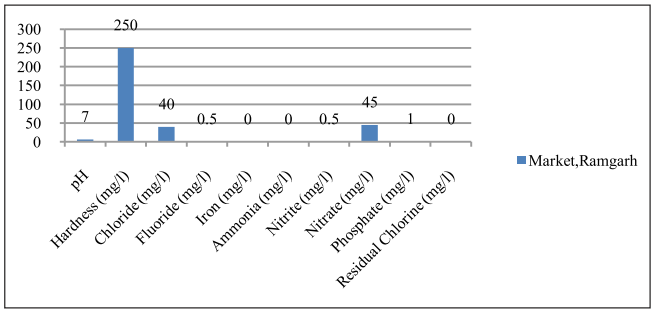


Figure 14:Groundwater quality at Market, Ramgarh.

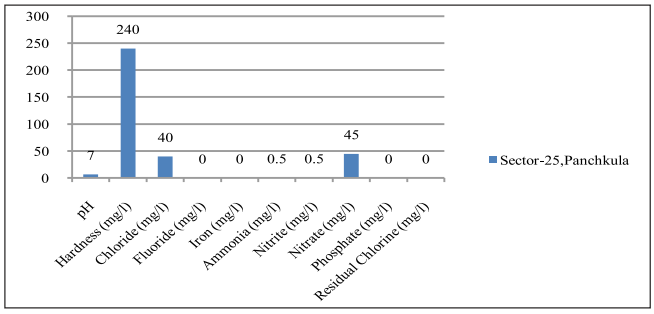


Figure 15: Groundwater quality at Sector-25, Panchkula.

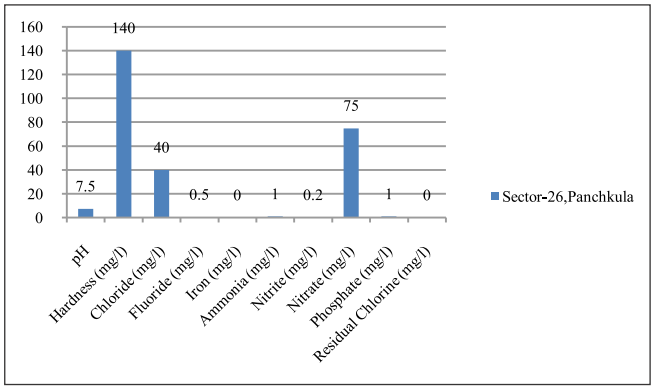


Figure 16: Groundwater quality at Sector-26, Panchkula.

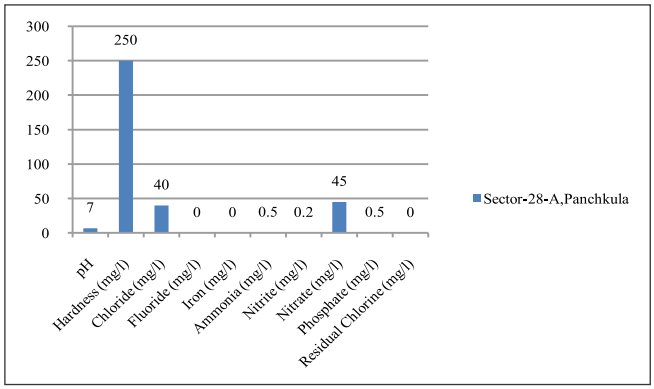


Figure 17: Groundwater quality at Sector-28-A, Panchkula.

desirable and hardness was permissible for drinking water purposes (Fig.17).

Groundwater quality at Sector-28-B, Panchkula

In groundwater sample collected at Sector-28-B, Panchkula,the chemical drinking water parameters pH, chloride, fluoride, iron, ammonia, nitrite, nitrate, phosphate, residual chlorine were found to be within desirable limit and hardness under permissible drinking water limit (Fig.18).

Groundwater quality at Sector-28-C, Panchkula

In groundwater sample collected at Sector-28-C, Panchkula, the drinking water parameters analyzed viz. pH, hardness, chloride, fluoride, iron, ammonia, nitrite, nitrate, phosphate, residual chlorine were under desirable limit (Fig.19).

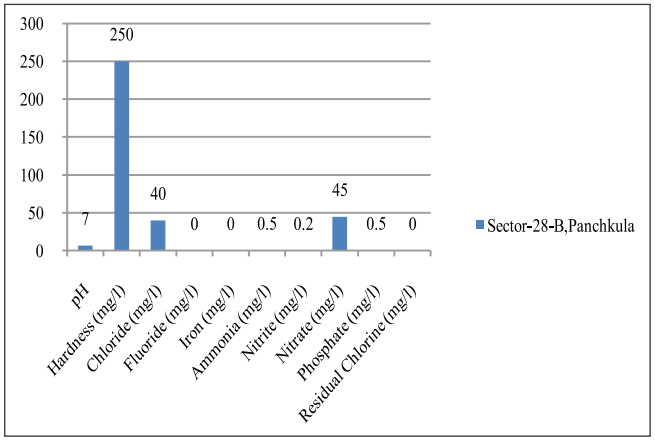


Figure 18: Groundwater quality at Sector-28-B, Panchkula.

Conclusion

In the study area, for all the eight groundwater samples pH, chloride, fluoride, iron, nitrite, phosphate, residual chlorine were in desirable limits for drinking purpose. Hardness was desirable at Govt. Primary School, Ramgarh (180 mg/l), Govt. Senior Secondary School, Ramgarh (100 mg/l),Sector-28,Panchkula (200 mg/l), Sector-26, Panchkula (140 mg/l) and permissible at Sector-28-A, Panchkula (250 mg/l), Sector-28-B, Panchkula (250 mg/l), Sector-25 (240 mg/l), Market, Ramgarh (250 mg/l). Ammonia was desirable atGovt. Primary School,Ramgarh (0.5mg/l), Govt. Senior Secondary School, Ramgarh (nil), Sector- 28-A, Panchkula (0.5 mg/l), Sector-28-B, Panchkula (0.5 mg/l), Sector-25, Panchkula (0.5 mg/l), Market Ramgarh (nil) and non-potable

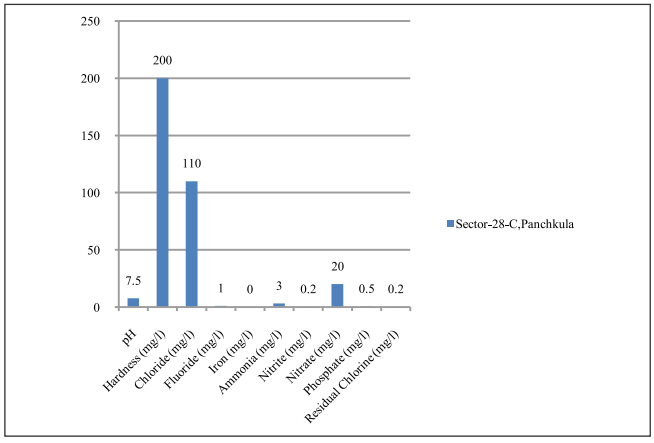


Figure 19: Groundwater quality at Sector-28-C, Panchkula.

at Sector-28-C, Panchkula (3.0 mg/l) and Sector-26,Panchkula (1 mg/l).Nitrate was desirable in seven groundwater samples and non-potable (75 mg/l) in one groundwater sample (Sector-26, Panchkula). Groundwater quality at Govt. Primary School, Ramgarh, Govt. Senior Secondary School, Ramgarh and Sector-28-C, Panchkula was desirable, at Market Ramgarh, Sector-25, Panchkula, Sector-28-A, Panchkula, Sector-28-B, Panchkula was permissible and Sector-26, Panchkula was found as non-potable. The data from the present study is highly useful for monitoring the groundwater quality in the study area and will serve as a reference for further related surveys.

References

1. **Abbulu Y, Rao GV.** A study on physico-chemical characteristics of groundwater in the industrial zone of Visakhapatnam, Andhra Pradesh. American Journal of Engineering Research (AJER). 2013;2(10):112-6.
2. **Agrawal R.** A study of physico-chemical parameters of groundwater quality of Dudu town in Rajasthan. Indian Journal of Remote Sensing. 2009;2(4):969-71.
3. **Deshpande SM, Aher, KR**. Evaluation of groundwater quality and its suitability for drinking and agriculture use in parts of Vaijapur, District Aurangabad, India. Research Journal of Chemical Sciences. 2012;**2 (1**):25-31.
4. **Madhav S, Ahamad A, Kumar A, Kushawaha J, Singh P, Mishra PK.** Geochemical assessment of groundwater quality for its suitability for drinking and irrigation purpose in rural areas of Sant Ravidas Nagar (Bhadohi), Uttar Pradesh. Geology, Ecology, and Landscapes. 2018; 2(2):127-36.
5. **Tripathi AK, Mishra UK, Mishra A, Tiwari S, Dubey P.** Studies of hydrogeochemical in groundwater quality around Chakghat area, Rewa District,Madhya Pradesh, India. International Journal of Modern Engineering Research.2012; **2** (6):4051-4059.
6. **Zidi C, Jamrah A, Al-Issai L.** Assessment of groundwater quality in Al-Buraimi, Sultanate of Oman. Journal of Materials and Environmental Sciences. 2017;**8** (4):1266-1276.

International Journal of Environment and Health Sciences (IJEHS)
2020, VOL. 2, NO. 3, 22–29
http://www.stenvironment.org/

STUDIES ON CONSORTIUM OF MARINE OIL DEGRADING BACTERIA FOR DEGRADATION OF HIGH SPEED DIESEL (HSD)

Namrata Kamble[1*], Chitra Pawar[1], Rajamani Mohanram[1] and Susan Titus[1]

Marine Biotechnology Department, Naval Materials Research Laboratory, Defence Research and Development Organisation, Ambernath (East), Mumbai 421 506, India.

Received on: 1.09.2020 **Revised on:** 30.09.2020 **Accepted on:** 10.10.2020

Abstract
Three bacterial strains namely RM1, RM2 and RM3 were isolated from oil polluted site of Mumbai harbor and were explored for growth on HSD as sole source of carbon and energy in four different combinations. Parameters investigated were growth of bacteria by optical density measurement along with viable count, production of bio-surfactant/ bio-emulsifier, its biochemical characterization and percent degradation of diesel by gravimetric method. Consortium A consisting of RM1 and Rm2 degraded 52.56% of HSD (4% v/v) in 7days employing 2% (v/v) of bacterial inoculum at room temperature on rotary shaker. Surface Tension of the Cell Free Filtrate (CFF) dropped from 68mN/m to<43mN/min all the cases indicating the ability of bacteria to produce extracellular surface active agents. The Emulsification Activity (EA24) of CFF prepared from culture broth of Consortium C and D was 100% with C8-C19 hydrocarbons whereas Consortium A exhibited 75-100% EA24with alkanes ranging from carbon number C10-C19.Results revealed that the oil degrading bacteria of consortia taken in this study had the potential to degrade hydrocarbons and could be further employed for remediation of HSD spillage.

Keywords
Biodegradation
Consortium
High Speed Diesel
Biosurfactant
Bioemulsifier
Emulsification Activity
Surface Tension

Introduction

Natural resources of oil are being exploited for fulfilling the energydemands of today's world. The dependency on transportation from one port to the other for supply increases along with the increase in demand(1, 2). In view of this, oil spills caused by accidental or anthropogenic activities in the sea causes great harm to the environment. The oil is either swept to farther areas, covers the surface or gets into the sediments after long run. It blocks adequate sunlight and also reduces the dissolution of oxygen, thus limiting the survival of natural flora and fauna in the aquatic ecosystem (3).

Several physico-chemical methods are used in response to oil spills. Physical methods involve use of barriers, booms, skimmers and absorbent materials to remove the overlaying surface oil. However, they barely succeed in complete removal of oil. Chemical methods involve the use of solvents or mixture of synthetic surfactants that breaks the oil into droplets. These droplets enter the water column and impart stress on the living inhabitants. These methods partially remove the oil from the environment and the persisting hydrocarbon (HC) & its components possess risk to the environment (2, 4).Although some of the oil contaminants are broken by photo-oxidation and evaporation, complete elimination depends on the metabolic activities of microbial population (5, 6).

Bioremediation is an eco-friendly strategy to deal with this menace. The ability of the bacteria to utilize the HCs has been documented since 1940s. This technology has been used to remediate marine oil spill since 1970s (3, 7). But the degradation capability varies with the change in chemical structure of the compound(8). This method is environment-friendly, low in cost, non-toxic and does not generate secondary pollution while the other methods are labor-intensive and usually expensive (7, 9, 10).

CONTACT *Corresponding author: knamrata93@gmail.com
Color versions of one or more of the figures in this article can be found online at www.stenvironment.org

Biodegradation is the breakdown of complex substances into simpler ones brought about by cellular enzymes. This brings about significant changes in the chemical structure of the organic pollutant resulting in the production of carbon dioxide, water and new microbial cellular constituents (biomass).The aerobic pathway leads to conversion of alkane chains into fatty acids, alcohol, aldehyde and carboxylic acids which are then channeled into the central metabolic process for β-oxidation.Single bacterium may not possess all the necessary biodegradative enzymes for breakdown of pollutants.Mixed microbial community acquire an array of catabolic genes and the cumulative effect of these gene products help in the degradation of complicated mixture of organic compounds present in the contaminated sites (11).

Hydrocarbons are insoluble in water; its solubility can be increased with the help of surface active agents (SAAs).Marine bacteria release Bio-surfactant (BS)/ Bio-emulsifier (BE) which facilitates the uptake of water-insoluble substrates present in the environment. BSs are amphipathic in nature comprising of hydrophilic and hydrophobic domains. The formation of micro-emulsions with the creation of micelle helps in solubilization of HC in water. This helps in reduction of Surface Tension (ST) of the system and allows the overlaying immiscible liquid to penetrate into water column (12).

Diesel oil is made up of n-alkanes (42.7%), cycloalkanes (33.4%) and aromatic (23.9%) HCs. Aliphatic HCs ranging from C_9 to C_{23} constitutes the major part. The utilization of such complex oil is shared by diverse microbial community. Some bacteria remain specialized in using a single type of substrate while some are capable of using a group of compounds (8, 13).

In an open sea, bioremediation is much more challenging due to constant fluctuating environmental conditions like tidal actions, changing seasons and transportation activities (6).Therefore, use of more than one bacterium will prove to be advantageous at the site of interest.

Over the years, studies on degradation of crude oil by bacteria have been reported throughout the world. But study on degradation of HSD by native bacterial consortia is limited (9). With relation to this, the present study focuses on testing combinations of marine oil degrading bacterial isolates from Mumbai harbor for its growth and utilization of HSD. It also aims on understanding the ability of bacterial strains for production of surface active agents for enhancing the bioavailability of insoluble HSD by bacteria.

MATERIALS AND METHODS

Bacterial consortia

Bacterial strains were isolatedfrom oil polluted site of Mumbai harbor. Isolation of oil degrading bacteria was carried out by enrichment technique (14). The three isolates were named as RM1, RM2 and RM3. Different combinations of three oil degrading bacteria were made as given below and used for this study (Table 1).

Table 1: Composition of bacterial consortia.

Consortium Name	Bacteria present
A	RM1 + Rm2
B	RM2 + Rm3
C	RM1 + Rm3
D	RM1 + RM2 + Rm3

Media preparation and inoculation

HSD was obtained from Naval Dockyard, Mumbai. The three bacterial strains were grown separately on Zobell Marine Agar (ZMA) plates. Single colony of bacteria was then inoculated into 100ml conical flask containing 50 ml of sterile Modified Bushnell Haas Broth (MBHB)with 1% HSD (filtered through 0.22µm membrane filter) and incubated at 37°C on a shaker incubator (Murhopye Scientific, India)at 180rpm for 24hrs.Optical Density (OD) of the culture medium was measured at 600nm using US-Vis Spectrophotometer (Labtek, India) (15). OD of culture suspension measuring 0.5 was used as inoculum. Aliquots of each culture suspension were added to give 2% (v/v) of mixed bacterial consortiain to 250ml conical flask containing 100 ml of sterile MBHB supplemented with 4% (v/v) HSD. The flasks were then incubated on a rotary shaker (Scigenics Biotech, India)at 180rpm at room temperature (RT). The experimental flasks were removed after 3rd, 5th and 7th day of incubation (each in triplicate along with control). Parameters to be studied were then carried out.

Viable count

The viable count of the cultures was determined by Spread plate technique. Serial dilution set was prepared using 0.89% of sterile saline in sterile1.5ml tubes. In 900µl of saline, 100 µl of culture suspension was added and vortexed. From this 100µl of aliquot was plated on sterile ZMA from respective dilution tube. The plates were incubated at 37°C for 24hrs. After incubation, the number of colonies were counted and using the below formula viable counts were found out.

Viable count (CFU/ml) = Number of colonies/ Volume of sample taken for plating in mL* Dilution factor

Gravimetric analysis

The weight of initial oil added (4ml) was 3.331gm. After termination on respective days, the broth was centrifuged at 15,000rpm at 4°C for 20mins and the upper layer of oil was collected into pre-weighed container. The percent degradation was found using the below formula:

% of oil degradation= Weight of remaining oil - Weight of initial oil added/ Weight of initial oil*100

pH change
The pH of the culture supernatant was measured using pH meter(Thermo Scientific, USA) after termination of growth.

Surface Tension measurement
The surface tension was determined by Wilhelmy plate method (16).The culture supernatant was filtered using 0.22µm membrane filter to remove any remnant cells from it to obtain a CFF. 10ml of CFF was taken from each flask in automated Surface Tensiometer (Kyowa, Japan). Sterile MBHB was used as Blank.

Emulsification Activity
Ea24 was determined by mixing 6ml of CFF with 0.6ml of HSD and vortex at high speed for 2mins. The tubes were kept standing and the readings were taken after 24hrs(17). EA24 was calculated using the below formula:

EA24(%) = Height of emulsified layer/ Height of Total layer*100 (Total layer= Height of Oil phase+ Emulsified phase)

Biochemical analysis
Folin-Lowry and Dubois methods were used to determine the protein and carbohydrate content of the CFFs respectively(18, 19).

Folin-Lowry method: Bovine Serum Albumin (BSA) (Sigma Aldrich, USA) was used as Standard. 1mg/ml of BSA was prepared in distilled water and used as working stock. Standard graph was plotted using concentration of BSA (ranging from 25-300µg/ml) vs. absorbance.

5ml of alkaline solution was added in a test tube with 1ml of CFF. It was kept at RT for 10mins. In this tube, 0.5ml of 1N Folin-Ciocalteau's phenol reagent (Sigma Aldrich, USA) was added, mixed and kept at RT for 30mins. Then OD was taken at 750nm using UV-Visible Spectrophotometer (LabIndia analytical, India).

Dubois method: D-glucose was used as Standard. 1mg/ml of D-glucose was prepared in distilled water and used as working stock. Standard graph was created using concentration of D-glucose (ranging from 25-300µg/ml) vs. absorbance.

In a test tube, 1ml of CFFwas added with 1ml of 5% phenol (Sigma Aldrich, USA) and vortexed. 5ml of concentrated Sulphuric acid was added to this and kept at RT for 10mins. Then OD was measured at 490nm using UV-Visible Spectrophotometer (LabIndia analytical, India).

RESULTS

Viable count&Optical Density

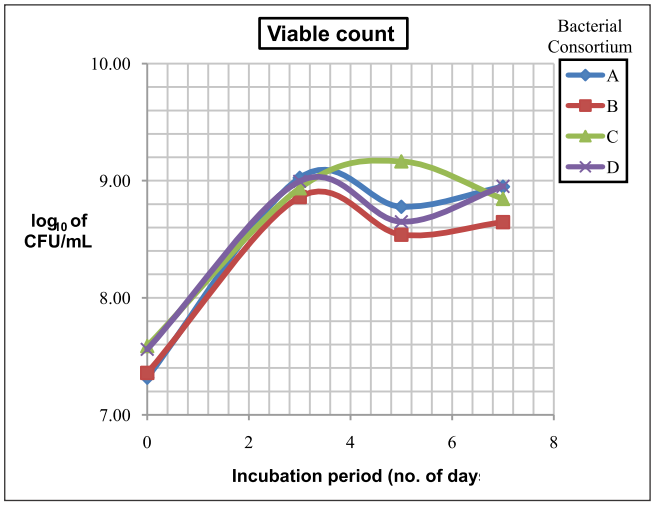


Figure 1: Graphical representation of viable count vs. incubation period.

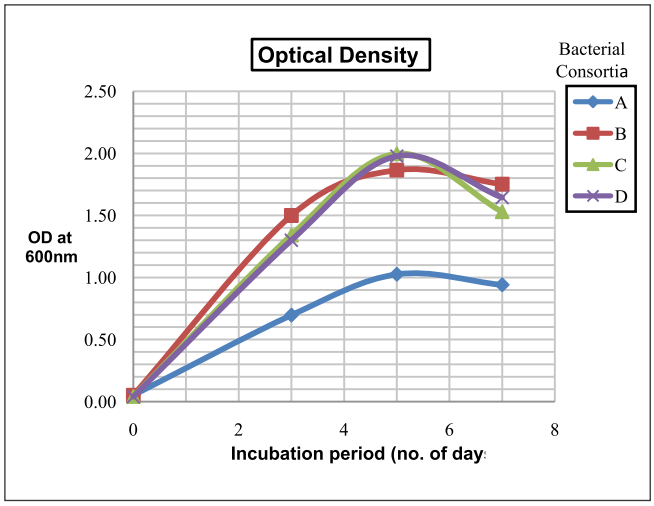


Figure 2: Graphical representation of OD vs. incubation period.

The culture was pre-grown in HSD to obtain actively growing bacteria to avoid prolonged lag-phase(15). Culture suspensions of 0.5 OD corresponding to 15 X10^5, 13.65 X 10^5 and 26.86 X 10^5 CFU/ml of RM1, RM2 and RM3 respectively, were taken to inoculate the flasks.

After 3 days of incubation viable count for consortia A, B and D exhibited growth of 11.5 X 10^8, 7.77 X 10^8 and 9.9 X 10^8CFU/ml, however consortia C exhibited further growth and a count of 14.8 X 10^8 CFU/ml was obtained after 5days of incubation. The viable count in all cases was found to remain more or less constant after this period (Figure 1).

OD of the culture medium was also determined at 600nm to monitor the growth of bacterial cells(9, 20, 21,22). It was found that the OD of consortia reached maximum (1.03, 1.86,

2.0 and 1.98 for consortia A, B, C and D respectively) after 5days of incubation. A common trend of increase in OD till 5days of incubation was seen after which it reduced (Figure 2).

3.2 Gravimetric analysis

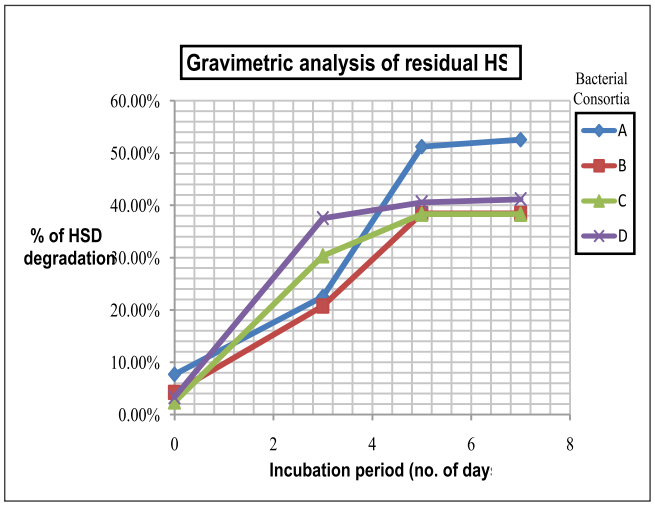


Figure 3: Graphical representation of percentage of HSD degradation.

Utilization of diesel oil was found to be more during the exponential phase of growth. It was seen that the bacteria in each combinations were able to utilize and degrade HSD. After 5days of incubation the bacteria in Consortium A, B, C & Ddegraded 52.56%, 38.46%, 38.31% & 43.07% of diesel oil respectively (Figure 3).

3.3 pH change

Table 2: Change in pH of broth of bacterial consortia.

Consortia	pH		
	Incubation period (no. of days)		
	3	5	7
CONTROL	7.295±0.01	7.23±0.0	7.13± 0.3
A	7.7±0.02	8.08±0.08	8.20±0.05
B	7.6±0.03	8.08±0.09	8.00±0.06
C	7.44±0.01	8.08±0.06	7.95±0.14
D	7.6±0.06	8.08±0.08	7.97±0.07

Average±SD, n=3

The initial pH of the broth was set at 7.5±0.2. The pH of culture broth increased slightly and turned alkaline for all the combinations as compared to control flasks of respective days (Table 2).

Surface Tension

Table 3: Surface Tension of CFF of bacterial consortia.

ST of CFF (mN/m)	Incubation period (no. of days)		
	3	4	5
A	40.20 ± 3.25	42.32 ± 1.13	38.83±1.27
B	42.37 ± 2.25	40.84±0.63	41.99±1.65
C	40.63 ± 3.80	37.03±0.73	39.06±0.93
D	40.82 ± 1.88	38.64±2.62	43.07±0.84

Average±SD, n=6

The surface tension reduction measurement of the CFF was recorded as a measure for release of SAA in the growth medium. For all the bacterial combinations the ST reduced from 68 mN/m (Blank) to <43mN/m in (Table 3).

Emulsification activity (EA24) with different hydrocarbons

Table 4: Emulsification Activity of CFF of bacterial consortia.

Hydro-carbon used	EA_{24} (%) by bacterial consortia			
C8-Octane	33.33± 3.0	66.66± 4.52	100.00± 2.4	100.00± 2.6
C9-Nonane	33.33± 2.4	33.33± 2.36	100.00± 3.12	100.00± 1.55
C10-n-Decane	100.00± 2.21	33.33± 1.25	100.00± 3.3	100.00± 2.31
C12-Dodecane	100.00± 1.8	33.33±1.72	100.00± 4.2	100.00± 3.0
C14-Tetradecane	100.00± 1.45	66.66± 2.69	100.00± 2.63	100.00± 2.8
C16- n-Hexadecane	75.00± 2.63	25.00± 2.48	100.00± 2.47	100.00± 3.32
C19- Pristane	100.00± 3.65	73.33± 4.2	100.00± 1.8	100.00± 3.5

Average±SD, n=3

Formation of emulsion between hydrophobic substrates and culture supernatantwas tested. Medium containing SAA produced by bacteria helps in penetration of oil molecules into the water layer. This enhances the availability of oil for uptakeby bacterial cells. EA_{24} determines the percentage of emulsion formation capacity and its stability, which was monitored till 24hrs.

The EA_{24} of CFF of Consortium C and D was 100% with carbon number alkanes ranging from C_8-C_{19}. Consortium B showed moderate EA(33-75%) with wide range of HCs. Consortium A did not exhibit good emulsion stability with short chain alkanes (C_8-C_9) however with medium and high carbon number alkanes (C_{10}-C_{19}) it was found to be stable (Table 4).

Biochemical analysis

Protein estimation

Table 5: Concentration of Protein in CFF of bacterial consortia.

Incubation period (no. of days)	Concentration of protein (µg/ml)			
	A	B	C	D
3	97.42 ± 3.79	138.68 ± 2.18	114.97 ± 2.18	127.35± 6.7
5	332.82 ± 12.23	404.56 ± 6.52	408.47 ± 11.22	387.74 ± 9.64
7	344.15 ± 3.16	312.00 ± 6.59	328.70 ± 4.58	293.58± 2.79

Average±SD, n=3

The CFF obtained from all combinations was examined for its protein and carbohydrate content by spectrophotometric method. Determination of biochemical content will help in understanding the nature of secreted metabolites. It was seen that in all the cases the protein concentration increased after 3days of incubation and thereafter after 5days it reduced slightly. For consortium A, protein concentration was 344.15µg/mlafter 7days of incubation. For consortia B and C protein concentration was 408.47&387.74µg/ml respectively after 5days of incubation period (Table 5).

Carbohydrate estimation

Table 6: Concentration of Carbohydrate in CFF of bacterial consortia.

Incubation period (no. of days)	Concentration of carbohydrate (µg/ml)			
	A	B	C	D
3	62.81±3.26	32.64±2.51	45.50±1.15	57.94±3.2
5	80.15 ± 0.83	38.84 ± 0.37	39.91 ± 2.90	49.10 ± 1.28
7	49.31 ± 3.45	55.66 ± 5.18	62.97 ± 1.02	51.08 ± 2.41

Average±SD, n=3

The concentration of carbohydrate in CFF was determined and found to be less than the protein content in all combinations of bacteria. For Consortium B and C, carbohydrate content was 50-60µg/ml after 7days of incubation, while for Consortium A it was ~80µg/ml after 5 days of incubation (Table 6).

DISCUSSION

The indigenous microorganisms inhabiting petroleum oil polluted marine environment are crucial for natural attenuation of hydrocarbons (HCs) during an oil spill. Oil degrading bacteria produce surface active agents which emulsify oil with water, resulting in increased bioavailability of these oil droplets for uptake and subsequent utilizationby bacteria for biodegradation (23).

Reported oil biodegradation studies revealed an initial increase in number of bacterial cells in response to sudden surplus in carbon levels (24).Increase in number of bacterial cells indicates survival of the introduced consortia in the presence of HCs (25). In our study also, the count of bacteria increased and indicated the ability of bacteria to grow in the presence of HSD. During the exponential phase of growth, the bacterial cells utilized diesel oil for reproduction and synthesis of cellular components. After the exponential phase, the bacterial load remained more or less similar. In agreement with our results, a study reported initial count of bacteria ranged from 10^4-10^5 CFU/ml and then hiked to $2X10^6$ and 10^7 CFU/ml in 10days of incubation period. The population remained fairly stable for rest of the study(24). Similarly, in another study performed with consortia on spent engine oil (1% v/v) gradual increase in viability was also observed only till 15days (4).

Turbidity of the growth medium was also measured in terms of OD to detect increase in cell numbers. The increase in OD indicated the knack of bacteria to grow in the presence of diesel oil by secreting requisite enzymes for its metabolism. It was able to utilize diesel as a sole source of carbon and energy. This may be due to increased bioavailability of the

HC to the bacterial cells. In a study, the OD reached 1.78±0.2 for consortia cultivated in 1% (v/v) of crude oil at 30°C after 7days of incubation. In the same study the OD of consortia was higher than that of individual strains (26). Increase in culture OD was reported till 5weeks after which it declined (9). In the present study, the viable count was maximum after 3days of incubation whereas maximum OD was obtained after 5days of incubation. Between day 3 and 5, the increase in OD might be due to the release of extracellular products into the medium and might be the reason for enhanced turbidity despite not much increase in viable count.

In this study, biodegradation percentage determination by gravimetric analysis revealed 52.56% diesel oil degradation by Consortium A within 7days of incubation period at RT. Diesel oil is composed of mainly saturated HCs having medium length chains(27). Therefore, these bacteria were considered to possess the ability of degrading medium length chains. In a similar study, seawater bioremediation trial reported more than 50% of degradation of Hcs components by consortium but within 30days of incubation period(6). The rate of diesel oil degradation was 82.65% after 12days of incubation with the help of both petroleum degrading bacteria as well as biosurfactant producing bacteria isolated from sea water (28).

Change in pH is known to affect growth of bacteria as well as the formation of emulsion. The emulsion creation capacity is seen to be lost at pH beyond 11 (28). pH7 was optimized for maximum crude oil degradation and growth of bacteria(1). In this study, the CFF of the all four consortium combinations showed a slight increase in pH which may be due to the alkaline chemical nature of the SAA or secondary metabolites or by-products of the catabolic reactions. Release of by-products from degradation of sludge oil post treatment with bacterial consortium for a period of 56days has been reported(30).

Surface Tension reduction value of CFF of all the four combinations of bacteria after 3days of incubation was below 43mN/m and reached till 37mN/m, this confirmed the production of SAA into the medium by the bacteria. A good BS producer reduces ST of medium by ≥ 20mN/m units as compared to distilled water(17). In present study, the ST of the culture medium reduced by >29mN/munits. This indicated good BS production capability of the bacteria used in this study. Reduction in ST of culture medium from 51.45 to 29.5mN/mwas achieved by bacterial consortium on degradation of crude oil after 3weeks of incubation(9). TERIK consortium showed drop in ST from 70mN/m to 34mN/m in 10days (31). In another study, mixed bacterial consortium was found to exhibit excellent growth on 1% Bombay High crude oil and was able to reduce the ST from 68mN/m to 34.1mN/m within 84hrs(32).In comparison with the reported studies, the percentage of HC used in our study was more (4%) and ST reduction was >25units as compared with control.

The presence of BS is necessary for production of emulsion between two immiscible liquids. If no surfactant is present, the two layers will separate after vigorous shaking. In the present study the emulsion was stable and did not separate. The Ea_{24} determined using different HCs in our study revealed the presence of extracellular surface active agents. EA ranged from 40-90% with different oils used with mixed bacterial consortium culture incubated for 70 days (33). In a study, consortium showed EA of 5±1 % with diesel oil (32). In the presence of HCs, the bacteria tend to secrete some biochemical molecules to emulsify the insoluble substrate and facilitate its transportation into the cell (32). In our study, reduction in ST may be attributed by extracellular material which comprised of more of proteins as compared to carbohydrates.

To accelerate the process of bioremediation treatment of oil contaminated site it is advantageous to use a mix of bacteria rather than using a single type of bacterium wherein the metabolic capacities of individual bacterium may result in synergistic action for degradation of fuel components (8, 32, 34, 35). It was also reported that effect of mixed bacterial population on crude oil degradation was better as compared to individual bacteria (26).Degradation of spent engine oil (1% v/v) was maximum (52%) as compared to individual bacterial strains after 10days of incubation under optimized conditions (4). Use of mixed culture over a pure culture imparts a benefit of displaying a collaborative influence between the bacterial cultures. One species may help in elimination of a toxic metabolite which might hinder the activity of other species. One may be able to degrade a compound partially then the other bacteria will degrade this partial product into a harmless end product (10). Crude oil has a complex composition. Degradation of this complex oil by bacterial consortium has been reported in several studies. A study reported 70% of crude oil degradation by bacterial consortium isolated from different contaminated sites of Aqaba region (Jordan) after 77days of incubation (36). High degradation rate of crude oil (1% v/v) was accomplished with the use of consortium than with the use of single bacterium for a period of 7days (26). In a study, a consortium comprising of five bacterial strains degraded 75.1% of crude oil (1% w/v) in 7days in marine environment. In the same study, 40-72% of crude oil degradation was achieved with consortium having three strains in different volumes. Not all the consortium of bacteria displayed good bioremediation action and the oil degradation rate was lower as compared to the single ones. Competition among bacteria of a consortium could be a cause for decreased bioremediation efficiency (7). However, in our study all the four combinations efficiently utilized diesel oil.

Use of carrier agents or immobilization technique; or addition of external nutrients is emphasized to enhance the degradation capability of bacteria. Bacterial consortium mounted on shell carrier along with nutrients showed 53.3% of total petroleum hydrocarbon degradation within 27weeks (2). Unlike in our study no such carrier agents or nutrients were used.

In open sea, bioremediation is much more challenging due to constant fluctuating environmental conditions like tidal action, variation in temperature, changing seasons and transport activities (6). Therefore, use of more than one bacterium may prove to be beneficial at the site of remediation.

Conclusion

This study investigated the performance of three oil degrading marine bacteria on degradation of high speed diesel. Various combinations of these bacteria taken for the study were found to utilize diesel as the sole source of carbon and energy and also produced extracellular surface active agents for facilitating the uptake of insoluble substrate.

References

1. **Muthuswamy Sathishkumar, Arthur Raj Binupriya, Sang-Ho Baik & Sei-Eok Yun**. Biodegradation of crude oil by individual bacterial strains and a mixed bacterial consortium isolated from hydrocarbon contaminated areas. 2008. Clean, 36(1): 92-96.
2. **Petra J. Sheppard , Keryn L. Simons, Eric M. Adetutu, Krishna K. Kadali, Albert L. Juhasz, Mike Manefielz, Priyangshu M Sarma, Banwari Lal & Andrew S. Ball.** The application of a carrier-based bioremediation strategy for marine oil spills. 2014. Marine Pollution Bulletin, Vol 84(1-2): 339-346.
3. **Antonio Dell'Anno, Francesca Beolchini, Laura Rocchetti, Gian Marco Luna & Roberto Danovaro**. High bacterial biodiversity increases degradation performance of hydrocarbons during bioremediation of contaminated harbor marine sediments. 2012. Environmental Pollution, Vol. 167: 85-92.
4. **Ganeshkumar, Vijayakumar L, Joshi G, Magesh Peter D, Dharani G &Kirubagaran R**. Biodegradation of complex hydrocarbons in spent engine oil by novel bacterial consortium isolated from deep sea sediment. 2014.Bioresource Technology, Vol 170: 556-564.
5. **Abdualdaim Mohammed Mukred, AidilAbd Hamid, AinonHamzah, Wan Mohtar& Wan Yusoff**. Development of three bacteria consortium for the bioremediation of crude petroleum-oil in contaminated water. 2008. Online Journal of Biological Sciences, 8(4): 73-79.
6. **Sharon N. Nuñal, Sheila Mae S. Santander-De Leon, Eugene Bacolod, Jiro Koyama, Seiichi Uno, Masayasu Hidaka, Takeshi Yoshikawa &HirotoMaed**. Bioremediation of heavily oil-polluted seawater by a bacterial consortium immobilized in cocopeat and rice hull powder. 2014. Biocontrol Science, Vol. 19(1): 11-22.
7. **Qingguo Chen, Jingjing Li, Mei Liu, Huiling Sun & Mutai Bao**. Study on the biodegradation of crude oil by free and immobilized bacterial consortium in marine environment. 2017. PLOS ONE| https://doi.org/10.1371/journal.pone.0174445.
8. **Lena Ciric, James C. Philp & Andrew S. Whiteley**. Hydrocarbon utilization within a diesel-degrading bacterial consortium. 2010. FEMS Microbiol Lett, 303: 116-122.
9. **Kaustuvmani Patowary, Rupshikha Patowary, Mohan C. Kalita & Suresh Deka**. Development of an efficient bacterial consortium for the potential remediation of hydrocarbons from contaminated sites. 2016. Frontiers in Microbiology, Vol 7, Article 1092.
10. **Abu Baker Salleh, Farinazleen Mohamad Ghazali, Raja Noor Zaliha Abd Rahman & Mahiran Basri**. Bioremediation of petroleum hydrocarbon pollution. 2008. Indian Journal of Biotechnology. 2: 411-425.
11. **Houghton J E & Shanley M S**. 1994. Chapter 1 Catabolic potential of Pseudomonads: a regulatory perspective. Biological Degradation and Bioremediation of Toxic Chemicals. G. R. Chaudhry. Dioscorides Press: Portland, Oregon. 11-32.
12. **N Qiao and Z Shao**. Isolation and characterization of a novel biosurafactant produced by hydrocarbon-degrading bacterium *Alcanivoraxdieselolei*B-5. 2009. Journal of Applied Microbiology, 108: 1207-1216.
13. **Yoon-Suk Kang & Woojun Park.** Protection against diesel oil toxicity by sodium chloride-induced exo-polysaccharides in *Acinetobacter* sp. strain DR1. 2009. Journal of Bioscience and Bioengineering. Vol. 109 (2): 118–123.
14. **Rajamani Mohanram, Chandrakant Jagtap & Pradeep Kumar**. Diversity among surface active agent producing marine oil degrading bacteria of Mumbai harbor. 2016. Marine pollution Bulletin. 105(1): 131-138.
15. **S Mnif, M Chamkha & S Sayadi**. Isolation and characterization of *Halomonas*sp. strain C2SS100, a hydrocarbon-degradaing bacterium under hypersaline conditions. 2009. Journal of Applied Microbiology, 107: 785-794.
16. **Thivaharan Varadavenkatesan and Vytla Ramachandra Murty**. Production of a lipopeptidebio surfactant by a novel *Bacillus* sp. and its applicability to enhanced oil recovery. 2013. ISRN Microbiology, Article ID 621519, 8 pages.
17. **Pia A. Willumsen & Ulrich Karlson**. Screening of bacteria, isolated from PAH-contaminated soils, for production of biosurfactants and bioemulsifiers. 1997. Biodegradation.7: 415-423.
18. **Lowry O H, Rosebrough J, Farr A L & Randall R J**. Protein measurement with the Folin phenol reagent. 1951. Journal of Biological Chemistry. 193: 265-275.
19. **Dubois M**. Colorimetric method for determination of sugar and related substance. 1956. Anal. Chem, 28(3): 350-356.
20. **Majid Zeinali, Manouchehr Vossoughi, Sussan K. Ardestani, Esmaeil Babanezhad and Mohamadreza**

Masoumian. Hydrocarbon degradation by thermophilic *Nocardiaotitidiscaviarum* strain TSH1: physiological aspects. 2007. *Journal of Basic Microbiology*. *47*: 534–539.

21. **Mojtaba Binazadeh, Iftekhar A. Karimi, Zhi Li.** Fast biodegradation of long chain *n*-alkanes and crude oil at high concentrations with *Rhodococcus*sp. Moj-3449. 2009. *Enzyme and Microbial Technology.* 45: 195–202.
22. **Pedro A. Isaza, Andrew J. Daugulis**. Ultrasonically Enhanced Delivery and Degradation of PAHs in a Polymer–Liquid Partitioning System by a Microbial Consortium. 2009. *Biotechnology and Bioengineering*, Vol. 104(1).
23. **Ibrahim M. Banat, Andrea Franzetti, Isabella Gandolfi, Giuseppina Bestetti, Maria G. Martinotti, Letizia Fracchia, Thomas J. Smyth & Roger Marchant**. Microbial biosurfactants production, applications and future potential. 2010. *Appl Microbiol Biotechnol.* 87: 427–444.
24. **Christoph Gertler, Daniela J. Nather, Simone Cappello, Gunnar Gerdts, Richard S. Quilliam, Michail M. Yakimov& Peter N. Golyshin**. Composition and dynamics of biostimulated indigenous oil-degrading microbial consortia from the Irish, North and Mediterranean Seas: a mesocosm study. 2012. FEMS Microbiol Ecol, 81: 520–536.
25. **Mittal & P. Singh**. A feasibility study for assessment of in-situ bioremediation potential of a crude oil degrading *Pseudomonas* consortium. 2009. *J. Sci. Res*, 2(1): 127-137.
26. **Zeynab Bayat, Mehdi Hassanshahian & Majid AskariHesni**. Enrichment and isolation of crude oil degrading bacteria from some mussels collected from the Persian Gulf. 2015. Marine Pollution Bulletin, Vol 101(1): 85-91.
27. **Huang Leia, XieJingb, Shi Xiaofengc & Lian Jingyan.** Research on the isolation, identification and degradation characteristics of a diesel oil degrading strain. 2013. Advanced Materials Research, Vol. 641-642: 206-210.
28. **Ke Shi, Jianliang Xue, Xinfeng Xiao, YanluQiao, Yanan Wu & Yu Gao**. Mechanism of degrading petroleum hydrocarbons by compound marine petroleum-degrading bacteria: surface adsorption, cell uptake and biodegradation. 2019. *Energy & Fuels, 33*(11): 11373-11379.
29. **David G. Cooper & Beena G. Goldenberg**. Surface-Active Agents from Two *Bacilllus* Species. 1986. Applied and environmental microbiology, Vol. 53 (2): 224-229.
30. **Pattanathu K S M Rahman, Thahira Rahman, P. Lakshmanaperumalsamy & Ibrahim M Banat**. Towards efficient crude oil degradation by a mixed bacterial consortium. 2003. *Bioresource Technology*, Vol 85(3): 257-61.
31. **Neha Sharma, Meeta Lavania, Vipin Kukreti & Banwari Lal**. Instigation of indigenous thermophilic bacterial consortia for enhanced oil recovery from high temperature oil reservoirs. 2020. PLOS ONE| https://doi.org/10.1371/journal.pone.0229889.
32. **Rahman K S M, Rahman T J, Lakshmana-perumalsamy P, Marchant R & Banat I M.** The potential of bacterial isolates for emulsification with a range of hydrocarbons. 2003. ActaBiotechnol. 23(4): 335–345.
33. **Krepsky N, Da Silva FS, Fontana LF & Crapez, MAC**. Alternative methodology for isolation of biosurfactant-producing bacteria. 2007. Braz. J. Biol., 67(1): 117-124.
34. **Wilfred F M Roling, Michael G Milner, D Martin Jones, Kenneth Lee, Fabien Daniel, Richard J P Swannell & Ian M Head**. Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient- enhanced oil spill bioremediation. 2002. Applied and Environmental microbiology, Vol. 68(11): 5537-5548.
35. **M Vinas, M Grifoll, J Sabate & AM Solanas**. Biodegradation of a crude oil by three microbial consortia of different origins and metabolic capabilities. 2002. Journal of Industrial Microbiology & Biotechnology, 28: 252-260.
36. **Emad I Hussein, Fuad A Al-Horani & Hanan I Malkawi**. Bioremediation capabilities of oil-degrading bacterial consortia isolated from oil-contaminated sites at the Gulf of Aqaba (Jordan). 2012. Biotechnology. 11(4): 189-198.
37. **Francesca Crisafi, Laura Giuliano, Michail M. Yakimov, Maurizio Azzaro& Renata Denaro.** Isolation and degradation potential of cold-adapted oil/PAH degrading marine bacterial consortium from Kongsfjorden (Arctic region). 2016.RendicontiLincei, Vol 27: 261-270.

B.
Health Sciences Section

International Journal of Environment and Health Sciences (IJEHS)
2020, VOL. 2, NO. 3, 32-36
http://www.stenvironment.org/

BIOCHEMICAL CONSTITUENTS OF MEDICINAL IMPORTANT *COLEUS FORSKOHLII* BRIQ. (PATHARCHUR), UNDER SOIL-MOISTURE STRESS CONDITION

N. A. Khan,[1]* G. S. Shaktawat,[2] S. D. Upadhyaya ,[2] A. Upadhyay[2]

[1]CSIR-National Environmental Engineering Research Institute, Delhi Zonal Laboratory,
[2]CSIR R & D Centre, A-93/94, Phase-1, Naraina Industrial Area, New Delhi -110028
Department of Plant Physiology, Jawaharlal Nehru Krishi Vishwavidalaya, Jabalpur, Madhya Pradesh.-482004

Received on: 28.12.2020 **Revised on:** 14.01.2021 **Accepted on:** 10.02.2021

Abstract
In the present study, *Coleus forskohlii* plant was subjected to different water stress conditions under controlled temperature in climate control Greenhouses. The roots of the plant were investigated for its biochemical parameters viz. forskolin, chlorophyll, proline and potassium percentage at variable water stress levels. The results revealed that the forskolin and proline content were found to be maximum in the roots of plants with 80% moisture stress. Similarly, the potassium content was also influenced by the water stress treatment. However, the chlorophyll content was found to be maximum in plants with 20% moisture stress. Consequent upon the interpretation of findings it can be concluded that the soil moisture stress has a significant influence on forskolin and proline content. Hence, the crop may be cultivated in a stress condition for maximum production of pharmaceutically important constituent i.e. forskolin.

Keywords
Biochemicals, soil-moisture stress, forskolin, proline, potassium.

Introduction

Coleus forskohlii Briq. (Patharchur) belongs to family Lamiaceae, is one of the significant medicinal herb having potential pharmaceutical properties. It is a mountain species occurring between the latitude of 31° N & 30° S in Palaetropic region. In India, it grows over a wide geographical range between the latitude 8°and 31°N in subtropical and warm temperate climate of lower Himalayas from Shimla Eastward to Bhutan and on the hills of central India. The plant grows wild in arid and semi-arid region of India and Thailand (1). It grows in 2 cm to 15 cm deep soil (pH-6.4-7.9) with deficient organic matter. In Egypt and Africa, the leaves of *C. barbatus* are used as an expectorant, emmenagogue and diuretic, while its foliage is employed in treating intestinal disorders.

The plant is cultivated commercially for its roots that are used to prepare drugs for hypertension, glaucoma, congestive heart-failure and certain type of cancer (2). Tuberous roots are succulent but hard, tortuous or straight, short or stout or long and slender. The roots are white or orangish pink flesh with bitter taste and are aromatic. The tuberous roots are found to be a rich source of forskolin. Forskolin is a major labdane diterpenoid occur in roots (3) and stem (4) along with deacetyl forskolin, 9-deoxyforskolin and 1,9-deoxyforskolin as the minor constituents.

Keeping the above facts in view the studies were conducted to evaluate the effect of various stress levels on Coleus and to work out the optimum moisture level for higher forskolin content in *Coleus forskohlii*. Finally, the growth and productivity of *C. forskohlii* at different moisture levels was also quantified.

Materials and Methods

Experimental Site

The present investigation was conducted at the Climate Control Greenhouses research area of medicinal and aromatic plants under Department of Plant Physiology, JNKVV, Jabalpur (M.P.) during the late Kharif season.

Experimental Detail

The experiment was carried out in a statically designed C.R.D. (Completely randomized design) with 4 replications

CONTACT *Corresponding author: na_khan@neeri.res.in
Color versions of one or more of the figures in this article can be found online at www.stenvironment.org

and 5 treatment levels. A total of 20 potted plants were used for the experiment and were planted. The variable treatments consist of five moisture levels i.e. 20% Moisture / 80% water stress, 40% Moisture / 60% water stress, 60% Moisture/ 40% water stress, 80% Moisture/ 20% water stress, 100% Moisture (Control)/ no stress.

Plant Material

The plants of *Coleus forskohlii* Briq. were transplanted in pot with one plant per pot. FYM, Biofertilizer and Inorganic Fertilizer were applied to each respective pot according to their required doses.

Sampling

Sampling was done at defined interval of 15 days i.e at 90, 110,130, 150 and 165 Days, and one plant was randomly selected from each treatment for growth analysis and biochemical estimation. The final harvesting was carried out on maturity and the plants were sun dried, roots were cleaned and analyzed for various yield components.

Determination of biochemical parameters

The effect of moisture stress was determined by evaluating the content of photosynthetic pigment and biochemicals including forskolin, proline and potassium in leaves and roots of plants at different growth stages. Leaf chlorophyll (a, b and total) content was estimated by CCM 200 (chlorophyll content meter). The potassium content was determined by photo-metrically using flame photometer (5). Free proline content in leaves was determined following the method given by Bates (6). The protocol was based on the formation of brick red coloured formazone by proline- ninhydrin complex in acidic medium, which is soluble in organic solvents like toluene and showed absorbance at 520 nm.

Analytical Method

Standard preparation

Weigh accurately 4 mg of forskolin reference standard was accurately weighed in a 10 ml volumetric flask and dissolved in methanol (stock solution).

Sample preparation

5 g of powdered root with methanol was taken in soxhlet unit and extraction was done at 50°C for about six hours. The resulting solution was filtered through a 0.45 µm nylon filter to remove all remaining undissolved material. The filtered solution was dried under vacuum and the resulting mass was further diluted in methanol.

HPTLC Method

5 µl each of test solution and reference solution were applied on five different tracks on precoated silica gel plate (10 x 10 cm) of uniform thickness (0.2 mm). The plate was developed using the Mobile phase Benzene: Ethyl Acetate (8.5 : 1.5) to a distance of 8 cm. The densitometry scanning wavelength was 210 nm for both reference and test solution track. Quantification of forskolin in the test solution was done by comparing their peak areas and Rf (0.27) with those present in the reference solution track. The post scanning visualization of spots was done by spraying the plate with p-anisaldehyde sulphuric acid reagent and heated at 110°C for 5 minutes.

Statistical analysis

Analysis of observation taken on different variables was carried out to know the degree of variation among all the treatments. The pooled data were statistically analyzed through Completely Randomized Design (8).

Results and Discussion

Chlorophyll content (cm^2)

The finding of the study clearly reveals the influence of moisture stress on plant biochemicals. The leaf chlorophyll was recorded from 90 days to 150 days of growth stages. The data for Chlorophyll content (g/m2) were statistically analyzed. It was found that the total leaf chlorophyll increases with the days of maturity and maximum content of chlorophyll was observed at 130 days of growth stages. It was further observed that the water-stress condition has a great influence on the leaf chlorophyll. The minimum Chlorophyll content was found in 80% moisture stress (9.198%) followed by 60 % (13.450%) and 40% moisture stress (16.100%) conditions. The maximum content was recorded in 20 % (18.119%) moisture stress condition. The results are given in Figure 1. Thus, the chlorophyll percentage in the leaves is directly proportional with the moisture content which clearly indicates the dependence of physiological phenomenon on moisture availability.

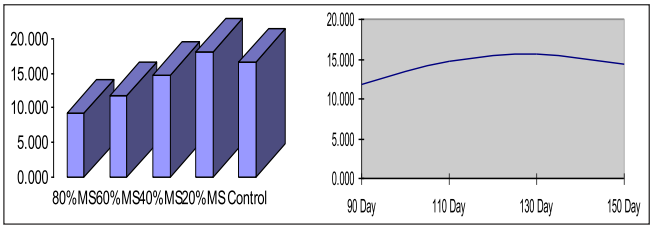


Figure 1: Chlorophyll content of *C.forskholii* at different moisture stress condition at different interval of days.

The potassium, proline and forskolin content in the roots of plant were very much influenced by growth stages and moisture stress conditions. The content of all the three biochemicals were recorded at different development stages and it was found that the content increases from 90 -150 days of growth and thereafter it decreases. The maximum content of potassium, proline and forskolin was recorded in the roots of plants at 150 days of growth stages.

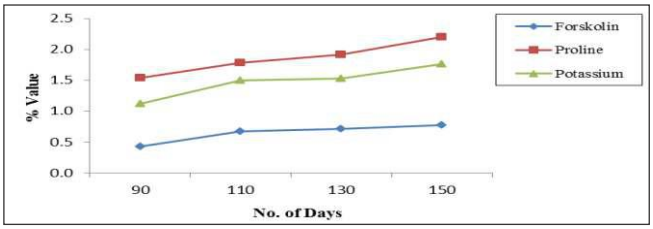


Figure 2: Forskolin, Proline and Potassium content of *C.forskholii* at different days of interval.

Potassium (%)

The data for potassium were analyzed and it was found that the water stress treatments have influenced on the potassium content. The maximum potassium content was found in 80% (1.7675%) moisture stress followed by 40% (1.55%) and 60% (1.49%) moisture stress conditions whereas minimum content was noted in 20 % (0.9675%) moisture condition (Figure 3). Potassium ion plays a central role in stomatal closure which is directly related with water stress tolerance mechanism. Therefore, it is one of the important biochemical parameter that is to be evaluated for water stress as it has signal responses in guard cells.

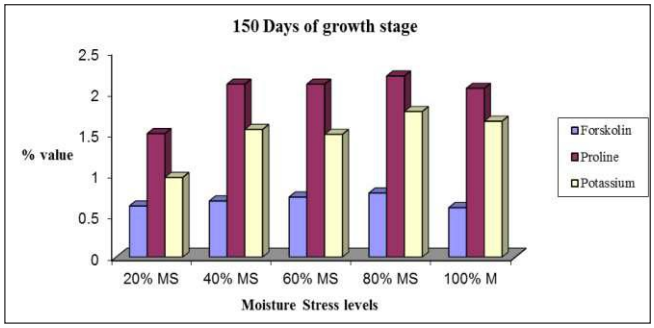


Figure 3: Forskolin, Proline and Potassium content of *C.forskholii* at different moisture stress condition.

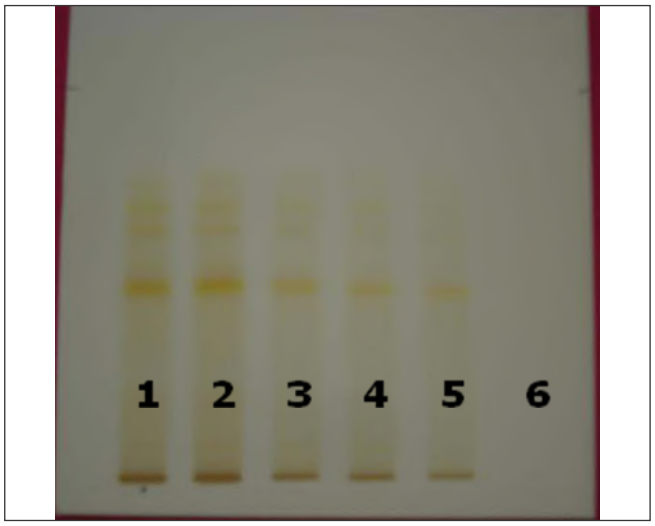


Figure 4 (a): TLC plate just after development. Lanes 1: 80% MS, 2: 60% MS, 3:40% MS, 4: 20% MS, 5-Control, 6: Standard.

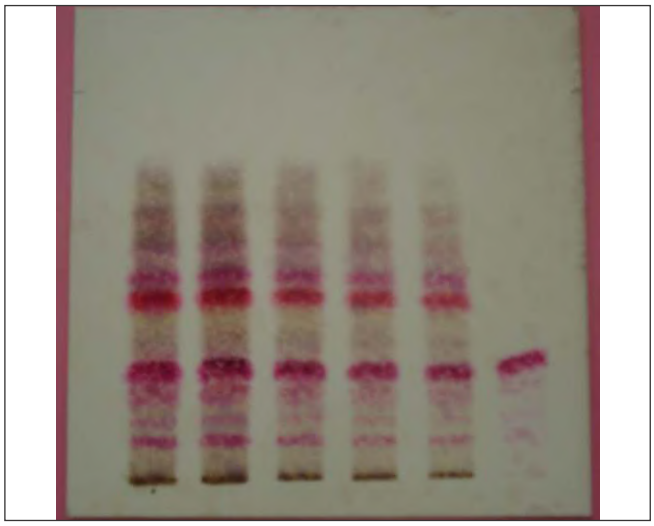
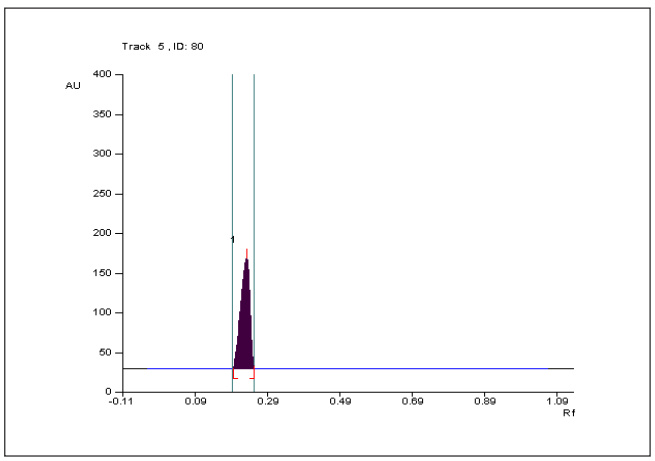
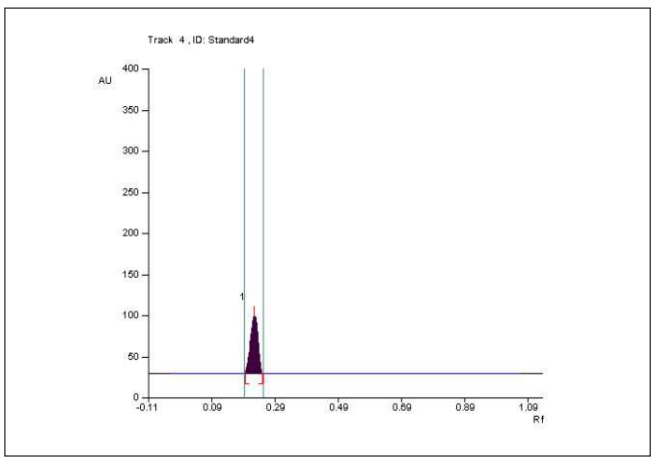


Figure 4 (b): Plate developed by *p*-anisaldehyde –sulphuric acid reagent.



Standard



Sample

Figure 4(c): HPTLC graphs of forskolin content in *C. forskholii*

Proline content (%)

Proline is an important amino acid produced under stress condition and is mainly related with the defense mechanism of plant. Proline content in relation to growth and water stress was noted up to 150 days. The data for proline content of roots for different water stress treatments were statistically analyzed. The results reveal the significant influence of all the water stress treatments on proline content. The maximum proline content was found in 80% moisture stress (2.2 %) followed by 60% and 40% moisture stress (2.1%) conditions. Whereas, minimum was recorded in 20 % (1.5%) moisture stress condition.

Proline content in relation to growth and water stress was noted up to 150 days. The results were in accordance with the reports given on the factors responsible for the accumulation of proline. The proline content of the leaf was determined at two development stages [7]. Study also revealed that the simulated accumulation of free proline in germinated wheat seeds and this metabolic manifestation may be used as a good biochemical index of moisture stress tolerance in plants [8]. Lab experiment which was carried out to assess the effect of PEG – 6000 induced short term moisture stress on drought tolerance of ten rice genotypes (9). The experiment was based on change in some important physico-chemical parameters like proline contents, RWC and NR activity in germinated seedling. Study also revealed that water stress induces accumulation of proline in the leaf [10]. After screening of a large number of sunflower genotypes, the results revealed that genotypes accumulating more proline did not show tolerance to moisture stress. In fact proline is considered as a water stress tolerance gene product, which may act to maintain cellular function through protection of cellular process by osmotic adjustments. Hence, understanding of proline is of great value in evaluating water stress tolerance mechanism and also to develop water stress tolerance varieties.

Forskolin content (%)

Forskolin, a labdane diterpenoid produced under stress condition. It is well related with growth and water stress mechanism of plant. The results of forskolin content in roots are depicted in Figure 3. The data for forskolin content in leaf was quantified through HPTLC (Figure 4a,b,c) and it was found that lowest forskolin was recorded in 20% moisture stress (0.621%) and control conditions, whereas 80% (0.778%), 60% (0.729%) and 40% moisture stress (0.681%) had the relatively high content.

The results were in accordance with the reports available for other plants. Andrographolide is the major and bitter constituent of *A. paniculata* Nees, extracted from the leaves of the plant (11). It is bicyclical diterpene lactones, increase under stress condition. The average forskolin content varied from 0.254% to 1.464% for dried Roots (12). Similarly, the date of planting as well as harvest dates has great influence on forskolin concentration of roots. Research reveals that the best planting period is during September/October. The plant is ready for harvest 41/2 to 5 months after planting. The plants are uprooted, tubers are separated, clean and sun dried for further use (13). Coleus plants raised in presence of arbuscular mycorrhizal fungi *Glomus bagyarajii*, showed an increase in plant growth and forskolin content over those grown in the absence of AM fungi (14). Forskolin and Proline content increases with increase in water stress condition. Potassium content is directly related to water stress treatment, thereby regulating the water stress tolerance mechanism in plants. Hence, the crop may be cultivated in a stress condition for maximum production of pharmaceutically important constituent i.e. forskolin.

Conclusion

On the basis of above interpretation of findings it can be concluded that the soil moisture stress has a significant influence on forskolin, proline and potassium content. Chlorophyll content of the leaves was greatly influenced by water stress condition. Forskolin and Proline content increases with increase in water stress condition. Potassium content is directly related to water stress treatment, thereby regulating the water stress tolerance mechanism in plants. Hence, the crop may be cultivated in a stress condition for maximum production of pharmaceutically important constituent i.e. forskolin.

Acknowledgments

The authors are thankful to the Head, Department of Plant Physiology, JNKVV to carry out this work. Present work is executed under the project entitled 'Niche area of excellence for the conservation, cultivation, processing and quality evaluation of medicinal and aromatic plants' funded by ICAR.

References

1. **Dubey MP, Srimal RC, Nityanand S, Dhawan BN**. Pharmacological studies on coleonol, a hypotensive diterpene from *Coleus forskohlii*. *Journal of Ethnopharmacology*. 1981; 3: 1-13.
2. **Gupta DP**. The Herbs: Habitate, Morphology and Pharmacognosy of Medicinal plants. 2008; 153-154.
3. **Bhat SV, Bajwa BS, Dornauer H, Desouza NJ, Fehlhaber HW**. Structure and stereochemistry of a new labdane diterpenoid from *Coleus forskohlii* Briq. *Tetrahedron Letter.* 1977; 19: 1669-1672.
4. **Saleem AM, Dhasan PB, .Rafiullah MRM.** Isolation of forskolin from stem of *Coleus forskohlii*. *Pharmacognosy Magazine*. 2005; 1: 89-92.
5. **Black CA**. Method of Soil Analysis. American Society of Agronomy Inc., Wisconsin, 1965.
6. **Bates LS, Waldren RP, Teare ID.** Rapid determination of free proline for water stress studies. *Plant and Soli*. 1973; 39: 205-208.
7. **Fisher RA, Kohn GD**. The relation of grain to vegetative growth and post flowering area in wheat crop under conditions of limited soil moisture. *Australian Journal of Agricultural Research.* 1996; 17: 281 – 295.
8. **Singh KP, Singh K.** Influence of simulated water stress on free proline accumulation in *Triticum aestivum L., Indian Journal of Plant Physiology*. *1983b;*26*:* 319-321.
9. **Inayatullah S, Irum R, Rehman A, Chaudhary MF, Mirza B.** Biological evaluation of some selected plant species of Pakistan. *Pharmaceutical. Biology*. 2007; 45: 397-403.
10. **Deshmukh PS, Shrivastava GC.** Variation in proline accumulation in sunflower genotypes under moisture

stress conditions. *Indian J Plant Physiology*. 1982; 25:307-309.

11. **Gorter MK.** The bitter constituent of *Andrographis paniculata* Nees. *Recueil des Travaux Chimiques*. 1911; 30: 151-160.
12. **Prathanturarug S, Soonthornchareonnon N, Chuakul W, Saralamp P.** Variation in growth and diterpene lactone among field- cultivated *Andrographis paniculata*. Journal of Natural Medicine. 2007; 61: 159-163.
13. **Rajamani K, Vadivel E. Marunthu Kurkan-** Medicinal Coleus. In: Naveena Mulikai Sagupaddi Thozhil Nuttpangal, Tamil Nadu Agriculture University, Coimbatore, 2009: pp.17-22.
14. **Sailo GL, Bagyaraj DJ** Influence of different Am-fugi on the growth, nutrition and forskolin content of *Coleus forskohlii*. *Mycological Research*; 109:795-798.

International Journal of Environment and Health Sciences (IJEHS)
2020, VOL. 2, NO. 1, 37-48
http://www.stenvironment.org/

FRACTIONS OF HIPPOPHAE RHAMNOIDES TURKESTANICA EXTRACT AND THEIR ADAPTOGENIC AND IMMUNOMODULATORY POTENTIALS

***Priyanka Sharma[a], Geetha Suryakumar[a], Himanshi Tanwar[b], Kshipra Misra[c], Lilly Ganju[a]**

[a]Defence Institute of Physiology and Allied Sciences, Lucknow Road, Timarpur, Delhi-110054, India
[b]National Institute of Immunology, Aruna Asaf Ali Marg, Delhi-110067, India
[c]Save the Environment, Heritage City, Gurugram, Haryana-122008, India

Received on: 07.01.2021 **Revised on:** 08.02.2021 **Accepted on:** 14.02.2021.

Abstract
Hippophae rhamnoides turkestanica (HRT)is capable of withstanding harsh climatic conditions prevalent at high altitude. Various medicinal and nutritional properties of the leaf-extract of this plant have already been established. *In vitro* studies of the extract ascertained the presence of various phyto constituents. In the present study adaptogenic and immunomodulatory potentials of different fractions of the whole leaf aqueous extract of HRT (SBT-5) are compared. This study was carried out on five fractions of SBT-5**.** Out of thefive fractions, polyphenolic rich fraction (PRF) showed 66.67% resistance against Cold-Hypoxia-Restraint(CHR) induced multiple stress at a four times lower dose compared to SBT-5. Supplementation of PRF significantly reduced the oxidative stress at tissues levels also. Successive Methanolic fraction amplified the immune response with Ovalbumin and Tetanus Toxoid antigens. As compared to SBT-5, PRF showed better antioxidant and adaptogenic potentials at four-time lower dose. This concludes that HRT fractions show improved performance under multiple stressful conditions as compared to the whole extract.

Keywords
Adaptogen, CHR, Phytoconstituents, Polyphenols, Stress.

Introduction

Globally known plant that is capable to with stand harsh environment prevalent at high altitude is seabuckthorn (genus *Hippophae*). *Hippophae rhamnoides turkestanica* (HRT) is an Indian seabuckthorn species growing wildly in North-West Himalayas (7000-15000 ft.). It is a dwarf to tall (3-15 feet), branched, and thorny nitrogen fixing deciduous shrub, native to Europe and Asia (Singh et al. 2013). Our laboratory has done extensive research on this plant and established the anti-oxidative, anti-stress, adaptogen (Geetha et al. 2003; Saggu et al. 2006;Sharma et al. 2015) and wound healing (Gupta et al. 2005) properties in whole leaves' extract of HRT. Although the whole extract worked in ameliorating the high altitude induced stress, its active principles/ compounds were yet to be identified. Broadly these compounds can be categorized into various groups, i.e., polyphenols, organosulphurs, carotenoids, alkaloids, and nitrogen-containing compounds (Roy M and Datta A 2019). Both *in vitro* and *in vivo* studies on this plant have proved its bio efficacy (Olas B *et al*. 2018).

In this study the extract is further fractionated to identify the potent fraction that works under the target conditions. Very little information is available on the relationship between active components in the leaves of seabuckthorn to those of their antioxidant and immunomodulatory roles. This article demonstrates the adaptogenic, immune activating and antioxidant potentials of different fractions of HRT obtained using various solvents with increasing polarity. To the best of our knowledge HRT fractions are studied for the first time for their multiple anti-stress activities.

Materials and Methods

Apparatus

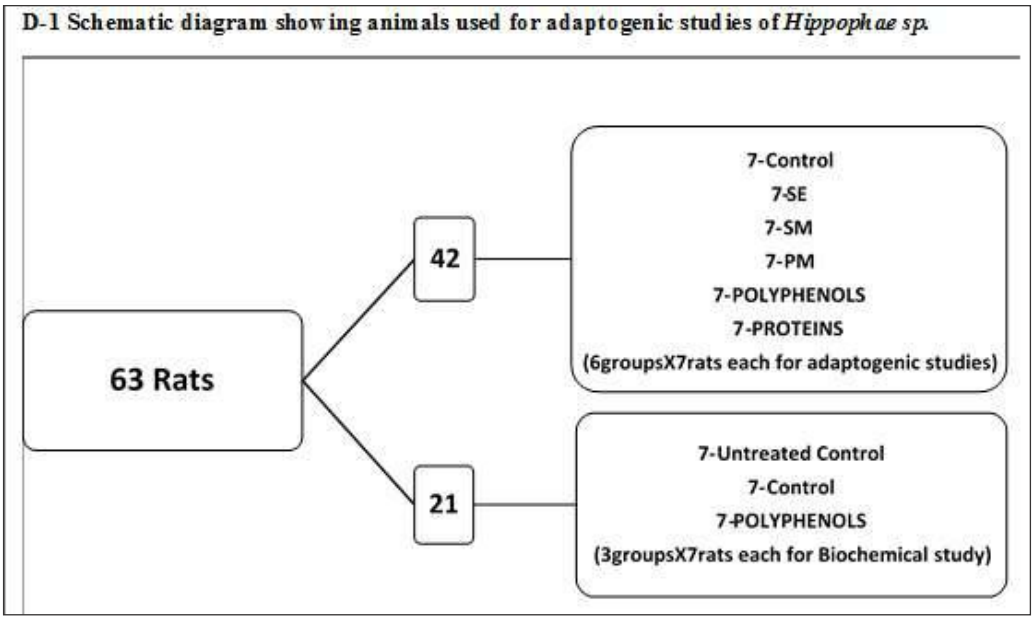
High Performance Thin Layer Chromatography (HPTLC), CAMAG, Switzerland; Cold-Hypoxia-Restraint Animal Model-CHR, Seven-Star, India ;rectal probes, Seven Star, India; Iso-thermex, Columbus Instruments, USA.

Plant Material and preparation of extract

Plant material with voucher specimen no. CSK HPKV-NAIP-SBT-Sp. T1 for *Hippophae rhamnoides turkestanica* (HRT) was obtained from CSK, Palampur, Himachal Pradesh and its

CONTACT *Corresponding author: priyankas@dipas.drdo.in
Color versions of one or more of the figures in this article can be found online at www.stenvironment.org

extract were prepared using Accelerated Solvent Extractor (ASE) (Sharma et al., 2015). The aqueous extract of HRT was code named as SBT-5.



Fractionation of aqueous extract of HRT
SBT-5 was subjected to following types of fractionation:

Fractionation with solvents of increasing polarity
1 gram of material was weighed and transferred to a boiling tube. 10 ml of hexane was added and sonicated for 15 minutes at room temperature (RT). The mixture was allowed to settle for 3 hours and then filtered. The same process was repeated with ethyl acetate and methanol. The yield was maximal with methanol and the fraction is coded as PM (i.e. Parallel Methanol) (Mohammad Azmin et al. 2016).

Successive extraction with the same solvents
1 gram of material was weighed and transferred to a boiling tube. 10 ml of hexane was added and sonicated for 15 minutes at RT. The mixture was allowed to settle for 1 hour and then filtered. The residue was again washed with hexane and then decanted. The same residue was dried and extracted with 10 ml ethyl acetate, sonicated for 15 minutes. The mixture was allowed to settle then decanted and the residue is again washed with ethyl acetate and after final decantation it was dried. The residue was finally extracted with methanol and processed in the same way as described above. Thus here 2 fractions were obtained. The decant of ethyl acetate was coded as SE (Successive Ethyl acetate) and that of methanol was coded as SM (Successive Methanol).The fractions were concentrated to dryness in water bath (temp. maintained 40°C) for 45 minutes (Roy et al 2018).

HPTLC Analysis
All the three fractions namely PM, SE and SM were subjected to HPTLC analysis. The mobile phase was Methanol, Chloroform, Ethyl acetate, Toluene and Formic acid in ratio 1.5:1:4.5:3:0.5. 10 µl of each fraction was applied on the TLC plate by Linomat sample applicator. The fractions were tested for their adaptogenic potential in CHR as described in later section on adaptogenic potential of fractions.

Out of the three fractions analysed, SM was found to possess maximum adaptogenic activity. Since it was obtained from a polar solvent (methanol) so further functional group separation technique (using TCA) was carried out and two additional fractions were obtained- one being Polyphenolic rich (PRF) and other Proteins rich. These two were also studied in CHR. HPTLC fingerprints for all the five extracts were obtained followed by derivatization with Ninhydrin and Ferric chloride solution.

Experimental design for evaluation of adaptogenic activity

Experimental animals
Sprague–Dawley inbred male rats, 12–14 weeks old, weighing 150 ± 10 gm, from the experimental animal facility of the Institute were used for the study. The rats were maintained inside polypropylene cages with free access to standard animal food pellets and water at 22 ± 1°C temperature, 55 ± 1% humidity and 12 h light–dark cycle. The study was approved by the Animal Ethical Committee of our institute in accordance with Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) of the government of India.

Adaptogenic Potential of Fractions
The schematic diagram (D-1) shows grouping of animals used for adaptogenic studies of aqueous extract of HRT (SBT-5).

Total 42 animals were used in the adaptogenic study using CHR. This model uses resistance in fall of rectal temperature as a measure of endurance (Ramachandran U et al. 1990). The rats were divided into 6 groups of 7 rats each. Out of the six groups, one group that served as control was administered with distilled water and rest of the five groups were administered with fractions at a dose 25 mg/kg body weight (0.5 ml for 150 gm of rat) 30 minutes prior to exposure in the CHR.The rectal probe was inserted 2 cm past the rectum of the rat and retained there with the help of adhesive plaster. The rectal temperature of the rats was monitored continuously, every minute by Iso-thermex temperature Recorder. Out of the five, the fraction that induced maximum physiological resistance (as shown by resistance in fall of rectal temperature) in rats to CHR induced hypothermia was further studied for its *in vivo* potential at tissue level. The results have been compared with those of SBT-5 as described in Sharma et al. 2015.

In Vivo Antioxidant status and stress markers

To determine mechanism of action,21 overnight fasted rats (D-1) were used to see the level of ROS, SOD, CAT, LDH, GSH/GSSG and MDA in the samples. The rats were divided into 3 groups of 7 rats each: (i) Untreated rats not exposed in CHR; (ii) Water administered rats i.e. Control and (iii) PRF administered rats at single dose of 25 mg/kg body weight. In both ii and iii groups oral administrations were done 30 minutes prior to exposure in CHR. The rest is same as described in section on *adaptogenic potential of fractions*.

Biochemical Assays

The methodology for biochemical assays are as described in Sharma et al., 2012 and Sharma et al.,2015. It is briefly explained here.

Lipid peroxidation

The reaction mixture containing TCA (trichloroacetate) and TBA (thiobarbituric acid) along with sample was boiled for 45 min in a boiling water bath. After cooling the supernatant was taken and the optical density (OD) was read at 531 nm to measure the amount of MDA formed in each of the samples. 1, 1, 3, 3-tetraethoxy propane was used as standard and the results are expressed as µM/ (mg protein).

Reduced Glutathione/ oxidizedglutathione (GSH/GSSG)

Ratio of reduced glutathione (GSH) and oxidized glutathione (GSSG) was obtained. A standard plot was obtained using 1mg/ml GSH and GSSG respectively and their respective concentrations in samples are extrapolated in mM/ml followed by their ratio.

Superoxide Dismutase (SOD)

The total SOD assay volume (3.0 ml) consisted of Tris-cacodylatebuffer, nitro blue tetrazolium salt (NBT), TritonX-100, water, sample and pyrogallol. A blank was run simultaneously consisting of water instead of sample. Enzyme kinetic activity was recorded at 540 nm for 3 min and change in OD per minute (Δ OD) was used to calculate % auto-oxidation inhibition to derive SOD units (U). One U of SOD was defined as 50% inhibition of the auto-oxidation caused by a certain volume of enzyme. The results of SOD activity have been expressed as U/(mg protein)$^{-1}$.

Lactate dehydrogenase (LDH)

Pyruvate buffer, sample and NADH were taken as reaction mixture. The LDH activity was calculated based on oxidation of NADH, using a molar extinction coefficient of $6.22\times10^{-3}M^{-1}cm^{-1}$.LDH activity has been expressed as nmol NADH oxidized per minute per mg protein [nmol min^{-1} (mg protein)$^{-1}$].

Catalase (CAT)

The reaction mixture consisted of buffer substrate and sample. Change in absorbance was recorded for 150s(every 15 s) at 240 nm. Catalase activity was calculated using an extinction coefficient of 0.041 cm (mmol)$^{-1}$ and expressed as mmol H_2O_2 consumed per minute per mg of protein [mmol min^{-1} (mg protein)$^{-1}$].

Total protein estimation

Copper reagent was added to 0.25 ml of sample. Tubes were incubated for 15min at 37°C. Folin-Ciocalteu reagent (FCR, 1N, 0.25 ml) was added and tubes were again incubatedat 37°C for 30 min. A standard graph was prepared using bovine serum albumin stock solution. Absorbance was read at 750 nm. The protein concentration in the test samples were extrapolated from the standard graph and expressed as mg ml^{-1}.

Reactive Oxygen Species (ROS)

Sample along with 2, 7-Dichloro-dihydro-fluorescein diacetate (DCFHDA) was incubated at room temperature for 15-45 minutes depending upon sample. Fluorescence was measured with excitation wavelength 485 nm and emission wavelength 530 nm against blank.

Immune response generated by fractions

Bioefficacy of fractions was evaluated *in vivo* using Balb/c mice (5–6 weeks old, weighing ±25grams) with one weak antigen Ovalbumin (ova) (Sigma) and one strong antigen Tetanus Toxoid (TT) from Kasauli, India, in terms of antibody titres generated against these antigens. The mice were primed with the formulations of antigen and fractions (10 µg dose/animal) on the day 1, followed by a booster on the day 21. On day 28, animals were bled for antibody (IgG) estimation by indirect ELISA. Each of the said doses was administered to the animals through intraperitoneal route. 2% Aluminum Hydroxide (Alum) and whole leaf extract of SBT (Alc-SBT) was used as positive controls (Tanwar et al., 2019).

Estimation of Immunoglobulin titers

For estimation of antibody titers, Microtitre plates, (Greiner, USA), were coated with OVA 20µg/ml in 0.01M PBS, pH 7.2) and TT (0.5µg/ml in 0.01M PBS, pH 7.2) for 24 hours at 4°C. All the incubations were carried out at 37°C with gentle

shaking and the plates were washed three times with 0.01% Tween20 ($PBST_{20}$) between the incubations. After thorough washing, plates were incubated for one hour with 1% BSA as a blocking solution, for avoiding any non-specific binding. Following thorough washing, plates were incubated for two hours with 1:10000 dilution of sera samples from immunized mice. Rabbit anti mouse IgG conjugated to horseradish peroxidase (HRP) (Sigma, Denmark), diluted in $PBST_{20}$ (1:4000 IgG) was added to the wells as secondary antibody. The enzyme reaction was visualized by incubation with Orthophenylene diamine (OPD) (Sigma, USA) substrate in (0.1M) citrate phosphate buffer with H_2O_2 (Sigma) as an oxidizing agent. The reaction was stopped after 10min by addition of 50µl of 2N H_2SO_4 (MP Biochemicals, USA) and the absorbance was measured at 450nm in an ELISA reader (BioTek Instruments, USA).

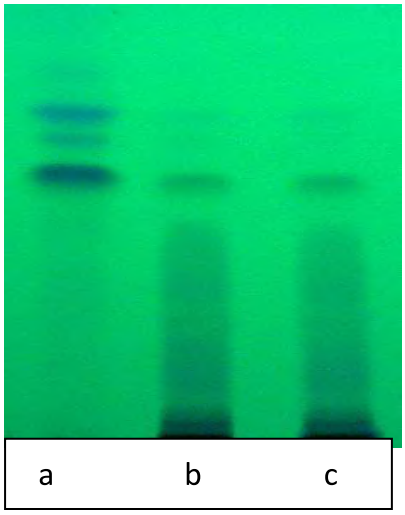
Statistical analysis

The results were analysed by one-way ANOVA (analysis of variance) with Sidak's Multiple Comparisons Test using Graph Pad Prism v.6. Differences were considered to be significant when the p values were < 0.05.

Results

HPTLC profiling of fractions of aqueous extract of HRT

Fig.1A shows TLC profiling of the three fractions, SE, PM and SM.Constituents' bands are seen in Histogram in Fig. 1B.



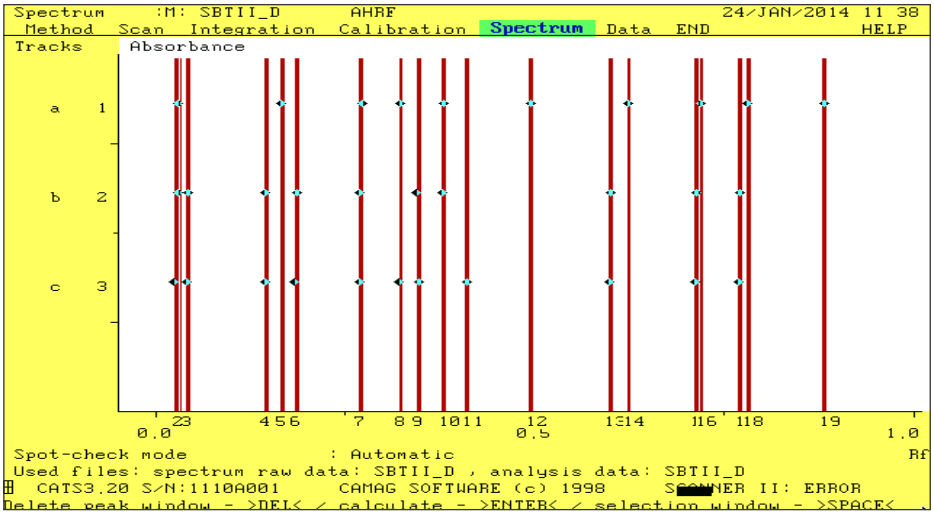


Figure1A:TLC photograph (under 254nm) of the Aqueous Extract

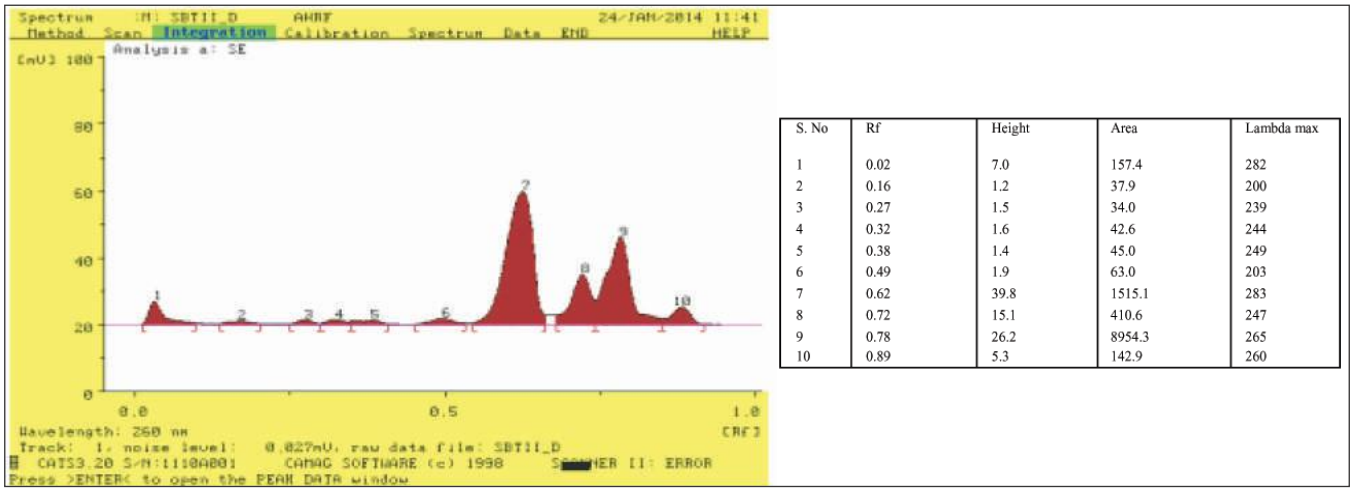
Track a– Successive ethyl acetate extract (SE)

Track b – Parallel methanol extract (PM)

Track c – Successive methanol extract (SM)

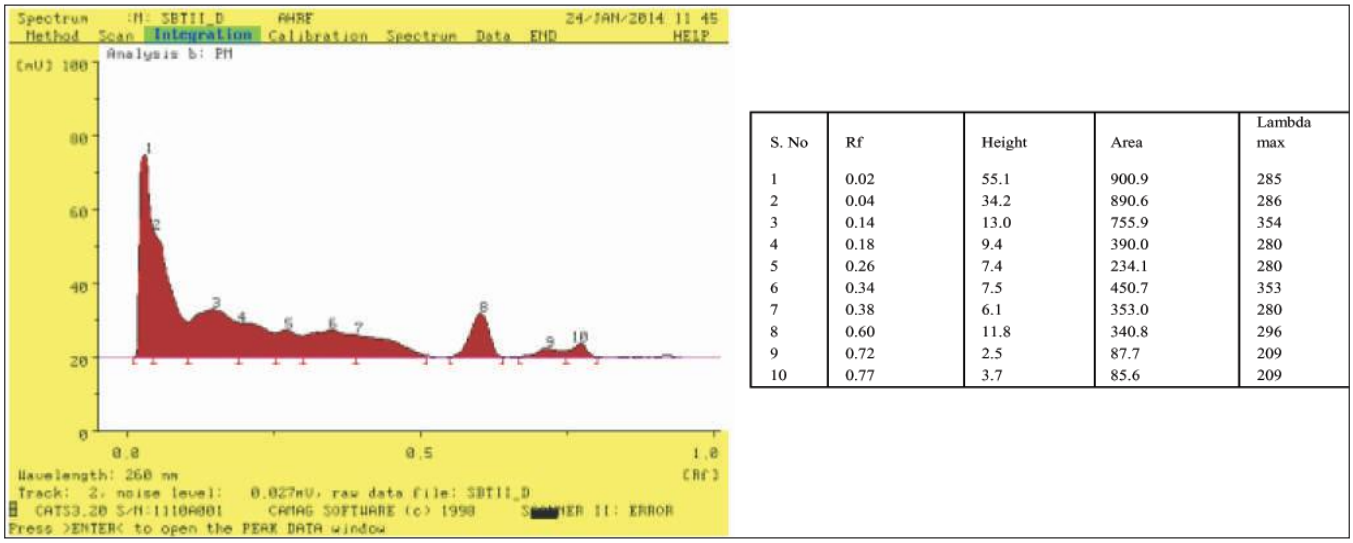
Figure 1B:Histogram of the Aqueous Extract.

HPTLC chromatograms for SE, PM and SM are shown in Fig 2A-2C with respectively 10, 10 and 11 unidentified components. The overall spectral scans (Fig.2D) shows overlapping peaks which state the possibility of presence of some common components in the three fractions. Fig. 3A-3B depict ninhydrin-derivatized TLC profiling of the fractions.



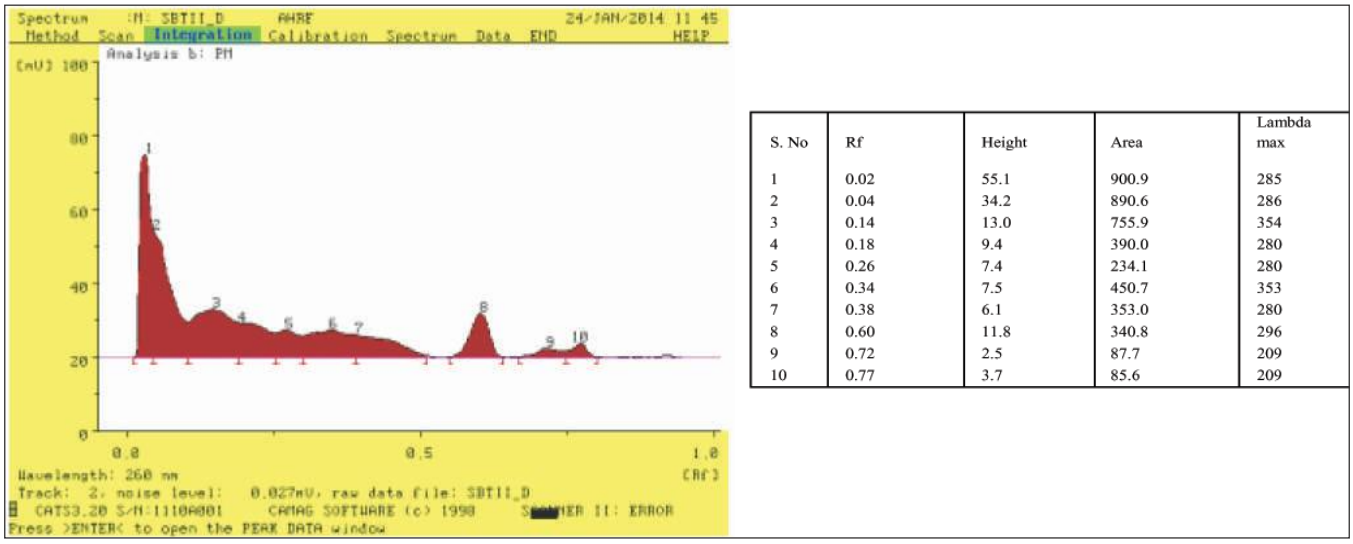
S. No	Rf	Height	Area	Lambda max
1	0.02	7.0	157.4	282
2	0.16	1.2	37.9	200
3	0.27	1.5	34.0	239
4	0.32	1.6	42.6	244
5	0.38	1.4	45.0	249
6	0.49	1.9	63.0	203
7	0.62	39.8	1515.1	283
8	0.72	15.1	410.6	247
9	0.78	26.2	8954.3	265
10	0.89	5.3	142.9	260

Fig. 2A: HPTLC chromatogram for Successive Ethyl acetate extracts (SE)



S. No	Rf	Height	Area	Lambda max
1	0.02	55.1	900.9	285
2	0.04	34.2	890.6	286
3	0.14	13.0	755.9	354
4	0.18	9.4	390.0	280
5	0.26	7.4	234.1	280
6	0.34	7.5	450.7	353
7	0.38	6.1	353.0	280
8	0.60	11.8	340.8	296
9	0.72	2.5	87.7	209
10	0.77	3.7	85.6	209

Fig. 2 B: HPTLC chromatogram for Parallel Methanol extracts (PM).



S. No	Rf	Height	Area	Lambda max
1	0.02	55.1	900.9	285
2	0.04	34.2	890.6	286
3	0.14	13.0	755.9	354
4	0.18	9.4	390.0	280
5	0.26	7.4	234.1	280
6	0.34	7.5	450.7	353
7	0.38	6.1	353.0	280
8	0.60	11.8	340.8	296
9	0.72	2.5	87.7	209
10	0.77	3.7	85.6	209

Fig. 2 C: HPTLC chromatogram for Successive Methanol extracts (SM).

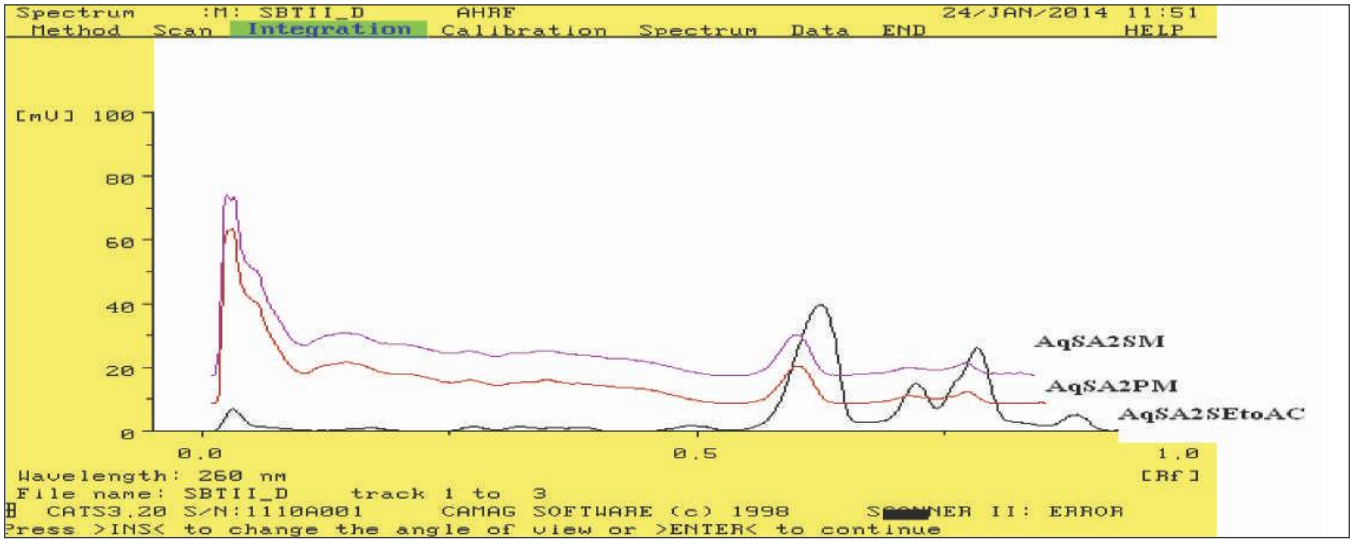


Fig. 2 D: Over all chromatograms.

Histogram of the extract after derivatization shows the presence of proteins in the three fractions.

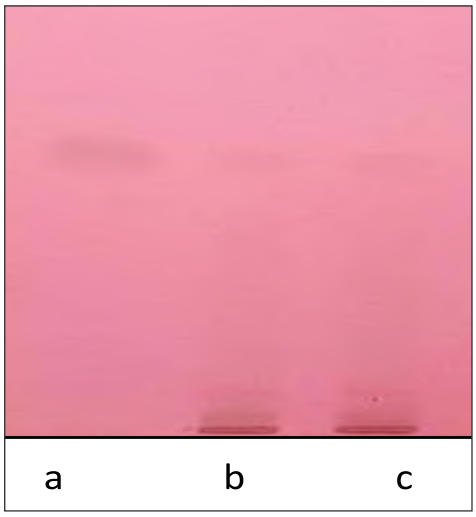


Figure 3A:TLC photograph (Ninhydrin-derivatized for Protein) of the Aqueous Extract
Track a – Successive ethyl acetate extract (AqSA2SE)
Trackb – Parallel methanol extract (AqSA2PM)
Track c – Successive methanol extract (AqSA2SM)

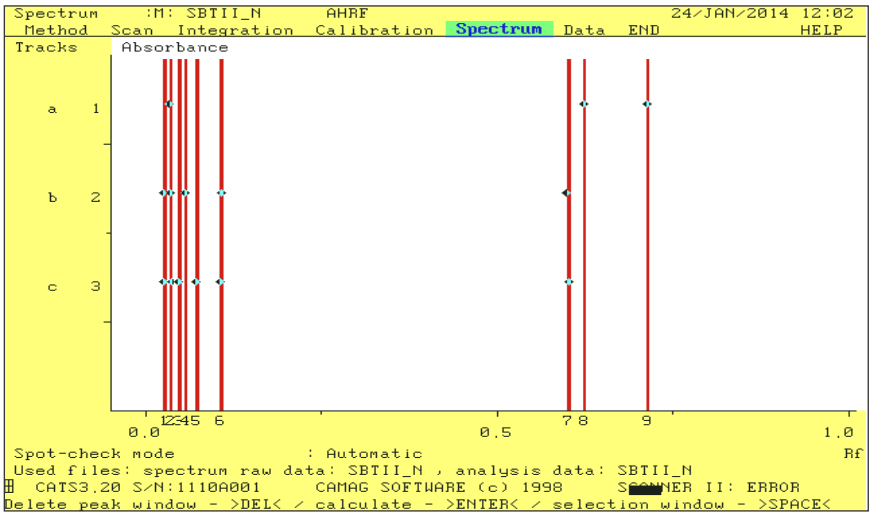
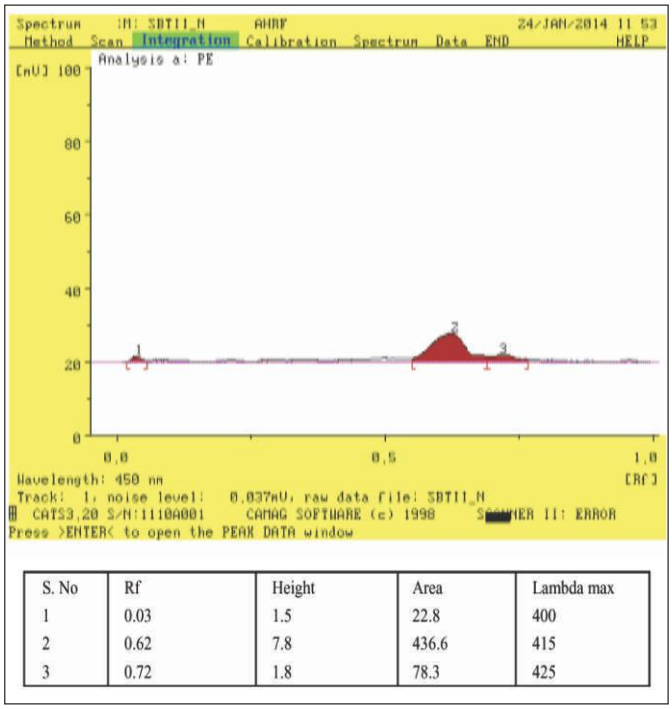


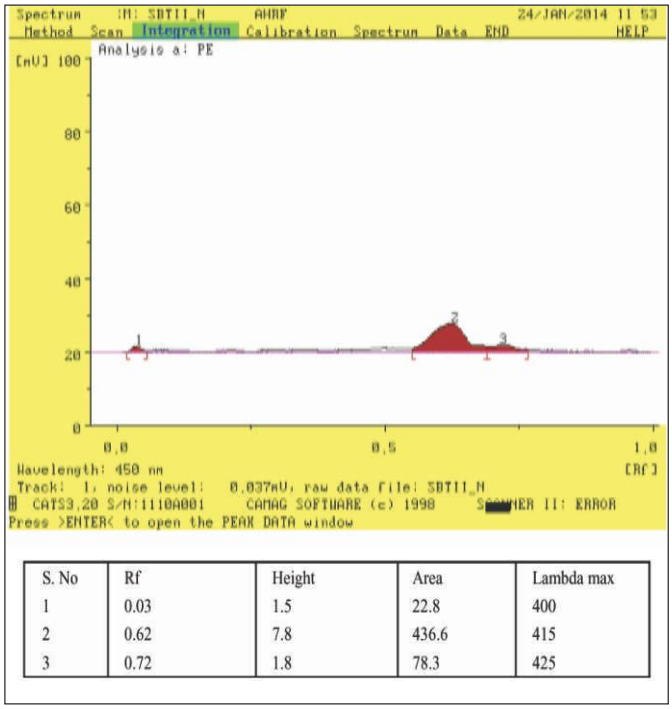
Figure 3B: Histogram for Water Extract after ninhydrin derivatization

Quantitatively 3, 5 and 6 different proteins are identified in chromatograms of the fractions SE, PM and SM respectively (Fig. 4A-4C). The overall spectral scan shows possibility of common proteins in the three fractions (Fig. 4D).



S. No	Rf	Height	Area	Lambda max
1	0.03	1.5	22.8	400
2	0.62	7.8	436.6	415
3	0.72	1.8	78.3	425

Fig. 4A: HPTLC chromatogram for Sucessive Ethyl aetate extract (AqSA2SE) after derivatised with Ninhydrin reagent.



S. No	Rf	Height	Area	Lambda max
1	0.03	1.5	22.8	400
2	0.62	7.8	436.6	415
3	0.72	1.8	78.3	425

Fig. 4B: HPTLC chromatogram for Parallel Methanol extract (AgSA2PM) after derivatization with Ninhydrin reagent.

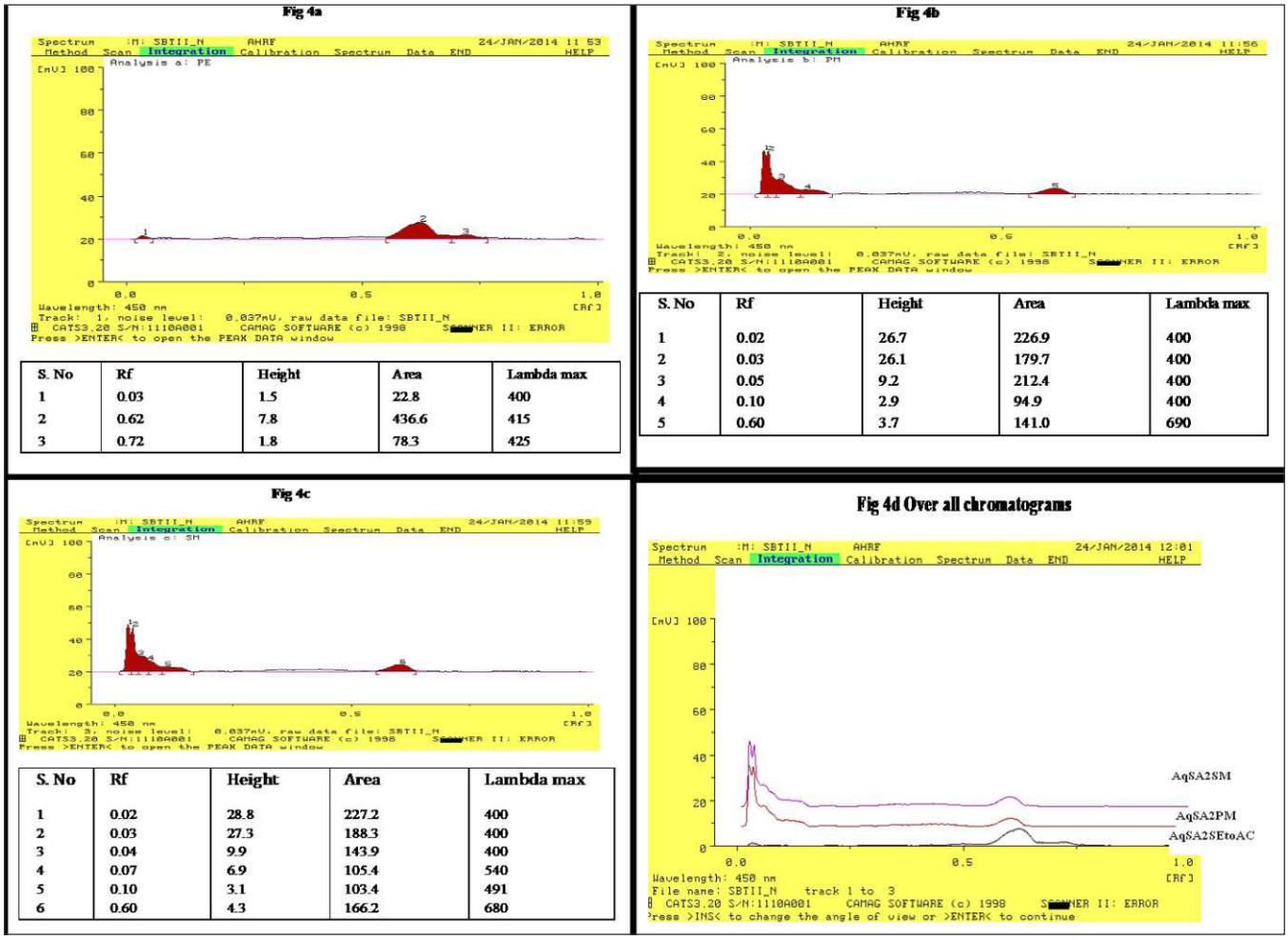


Fig 4a

S. No	Rf	Height	Area	Lambda max
1	0.03	1.5	22.8	400
2	0.62	7.8	436.6	415
3	0.72	1.8	78.3	425

Fig 4b

S. No	Rf	Height	Area	Lambda max
1	0.02	26.7	226.9	400
2	0.03	26.1	179.7	400
3	0.05	9.2	212.4	400
4	0.10	2.9	94.9	400
5	0.60	3.7	141.0	690

Fig 4c

S. No	Rf	Height	Area	Lambda max
1	0.02	28.8	227.2	400
2	0.03	27.3	188.3	400
3	0.04	9.9	143.9	400
4	0.07	6.9	105.4	540
5	0.10	3.1	103.4	491
6	0.60	4.3	166.2	680

TLC profiling in Fig 5A-5B depicts bands for polyphenolic rich compounds after derivatization with Ferric chloride solution. Fig 5B shows the Histogram of derivatized HPTLC plate for the constituting poly phenols in the three fractions.

Respectively 5, 2 and 3 phenolic rich compoundsare identified in SE, PM and SM (Fig. 6A-6C). As such no overlapping peaks has been identified (Fig 6D).

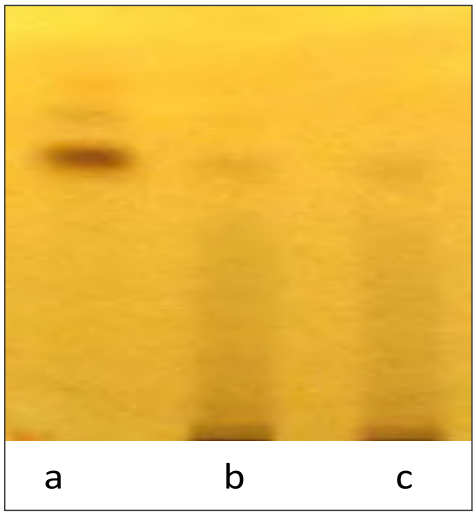


Figure 5A:TLC photograph (Ferric chloride derivatization for Poly Phenol)
Track a – Successive ethyl acetate extract (AqSA2SE)
Trackb – Parallel methanol extract (AqSA2PM)

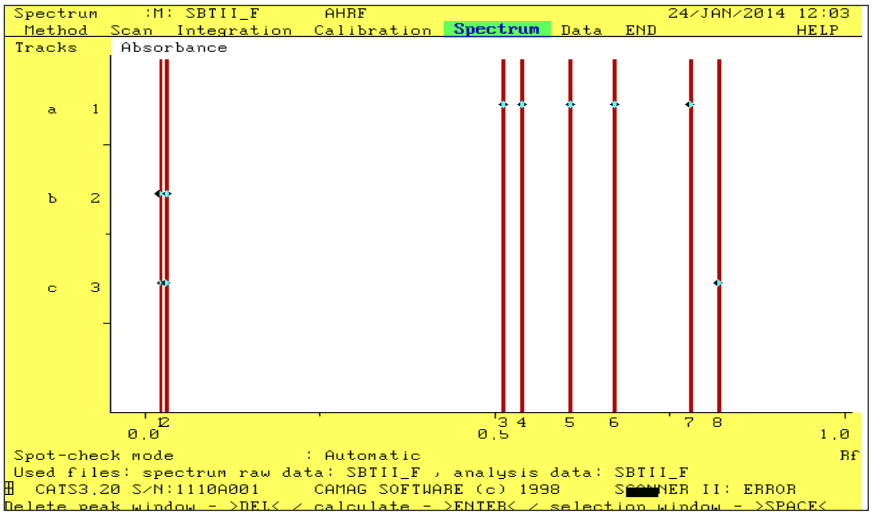
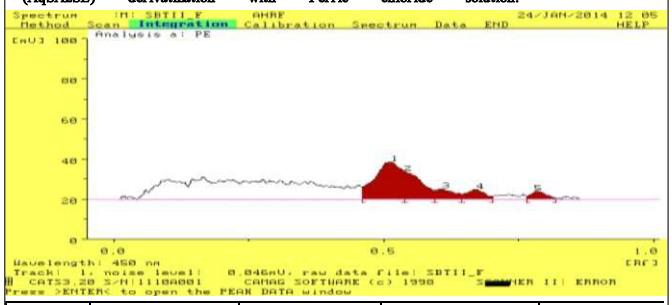


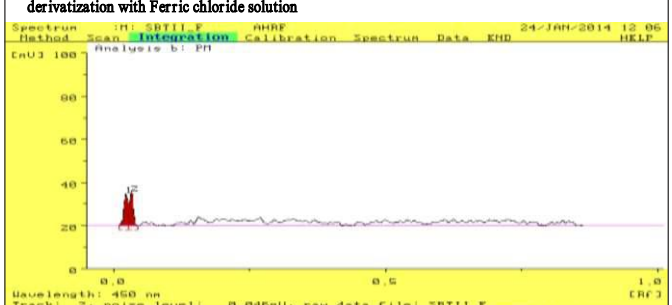
Figure 5B: Histogram for Water extracts derivatization with Ferric chloride solution

Fig.6A. HPTLC chromatogram for Successive Ethyl acetate extract (AqSA2SE) derivatization with Ferric chloride solution.



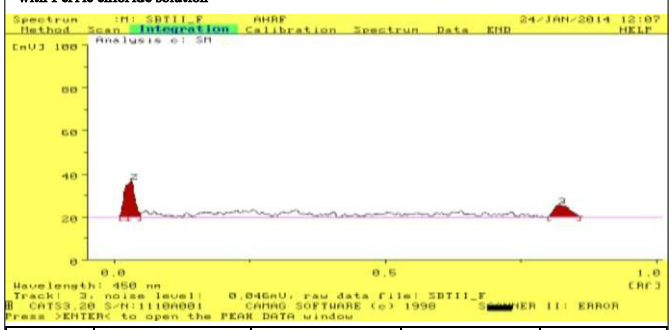
S. No	Rf	Height	Area	Lambda max
1	0.51	18.6	801.2	418
2	0.54	13.8	382.9	415
3	0.61	5.2	149.5	404
4	0.67	4.9	139.32	400
5	0.78	3.9	97.0	400

Fig.6B. HPTLC chromatogram for Parallel Methanol extract (AqSA2PM) derivatization with Ferric chloride solution



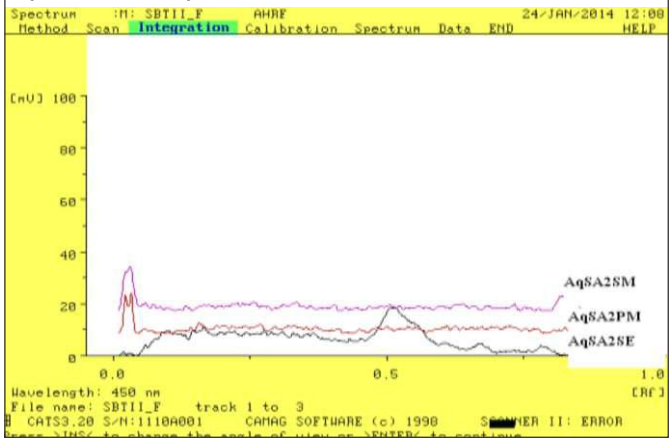
S. No	Rf	Height	Area	Lambda max
1	0.01	14.9	96.1	604
2	0.02	15.6	93.2	600

Fig.6C. HPTLC chromatogram for Successive Methanol extract (AqSA2SM) derivatization with Ferric chloride solution



S. No	Rf	Height	Area	Lambda max
1	0.002	15.2	115.2	600
2	0.02	16.9	156.8	503
3	0.82	6.3	211.9	400

Fig.6D. Over all chromatograms:



Adaptogenic activity of seabuckthorn fractions

The adaptogenic potential of the five fractions of SBT-5 was studied for their adaptogenic potential using CHR animal model. The results were compared both with control and SBT-5 (Fig.7). SBT-5 was given as 100 mg/kg body weight (Sharma et al. 2015)p.o whereas the fractions were given as 25 mg/kg. body weight.

As given in Table-1, compared to control, SE showed 43.48 % resistance against CHR induced hypothermia and a faster recovery by 10.43%. SM showed a maximum, 69.57% resistance against CHR induced stress and the time taken to attain T_{rec}37°C reduced by 9.20%. An increase of 34.78% and 9.20% in time taken to attain T_{rec}23°C and T_{rec}37°C respectively was observed for PM. The Polyphenolic rich fraction (PRF) of SBT-5 showed the maximum adaptogenic potential. It showed

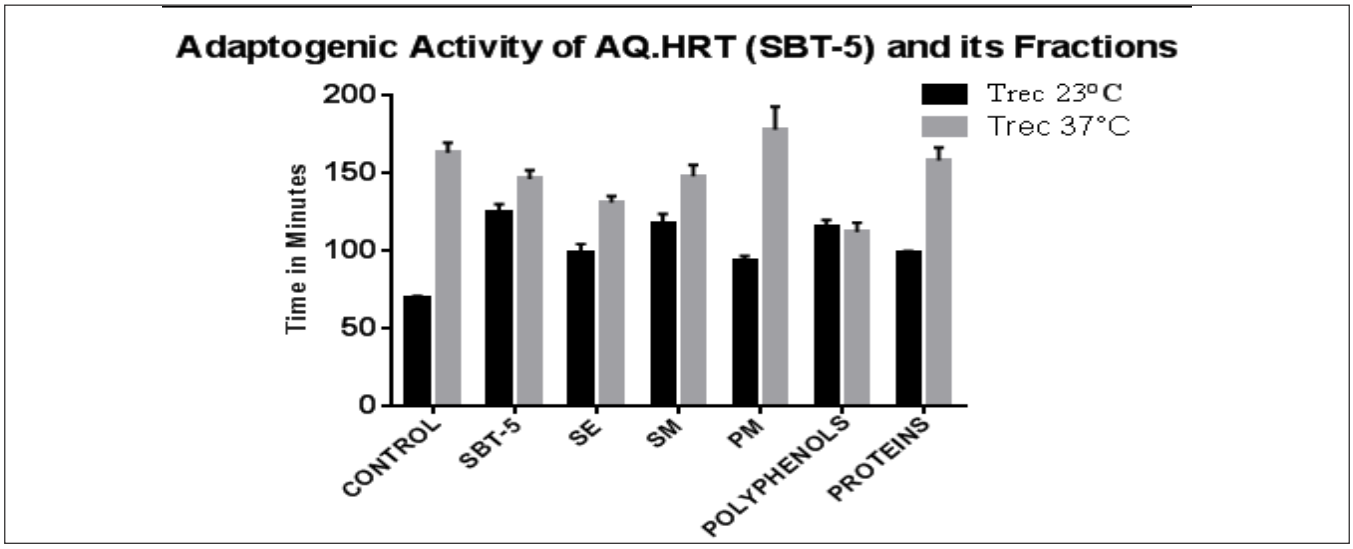


Figure 7:Adaptogenic activity of Seabuckthorn Fractions with comparison to Control & SBT-5.

a hypothermic resistance of 66.67% and a faster recovery from hypothermia by 31.29%. The resistance of 42.03% from CHR induced hypothermia was seen in Proteins fraction whereas the recovery was fastened only by 3.07%. In all these results the %change in time taken to attain T_{rec} 23°C was significant whereas the %change in time taken to attain T_{rec} 37°C was non-significant except in PRF.

The same results of the fractions as detailed above when compared to SBT-5, they were found to be comparable for SM and PRF where a non-significant change of 6.40% and 8% were observed for resistance against CHR induced hypothermia whereas for SM the % change from recovery from hypothermia was a non-significant 1.37% only, it was significantly high for PRF at 23.29%. For the rest of the fractions the resistance from CHR induced hypothermia was 20.08, 25.6 and 21.6% respectively for SE, PM and Proteins and the delay in the time taken for recovery were 10.27, 21.92 and 8.22% respectively.

Table-1: Adaptogenic activity* of seabuckthorn leaf extract (SBT-5) and its five fractions after single dose (p.o.) of 100 and 25 mg/Kg. body weight respectively.

Extracts	T1[a]	T2[b]
CONTROL	69±1.88	163±6.64
SBT-5	125±4.97*	146±5.89
SBT SA II-SE	99±9.02	131±4.17
SBT SA II-SM	117±6.67	148±7.27
SBT SA II-PM	93±3.52	178±14.83
CARS-II-POLYPHENOLS	115±4.67	112±6.02
CARS-II-POLYPHENOLS	115±4.67	112±6.02
CARS-II-PROTEINS	98±1.67	158±8.34

*Values are mean ± SE of six rats in each group; T1[a]: time taken (in min) to attain Trec 23°C; T2[b]: time taken (in min) to attain Trec 37°C; *P<0.05, compared with respective group of control rats.

Antioxidative status and stress markers

The results on antioxidant status and stress markers in the blood samples of rats are shown in Fig.8 (a-f).

In blood samples there was 171% increase in the fold change of ROS activity in exposed control rats as compared to unexposed control. However, the levels reduced by a non-significant percent of 18% for PRF. The SOD levels were increased by 119% in exposed control rats as compared to unexposed control. However, the levels were significantly reduced by 51% for the fraction as compared to exposed control. After administration of SBT-1 and 5the level of CAT

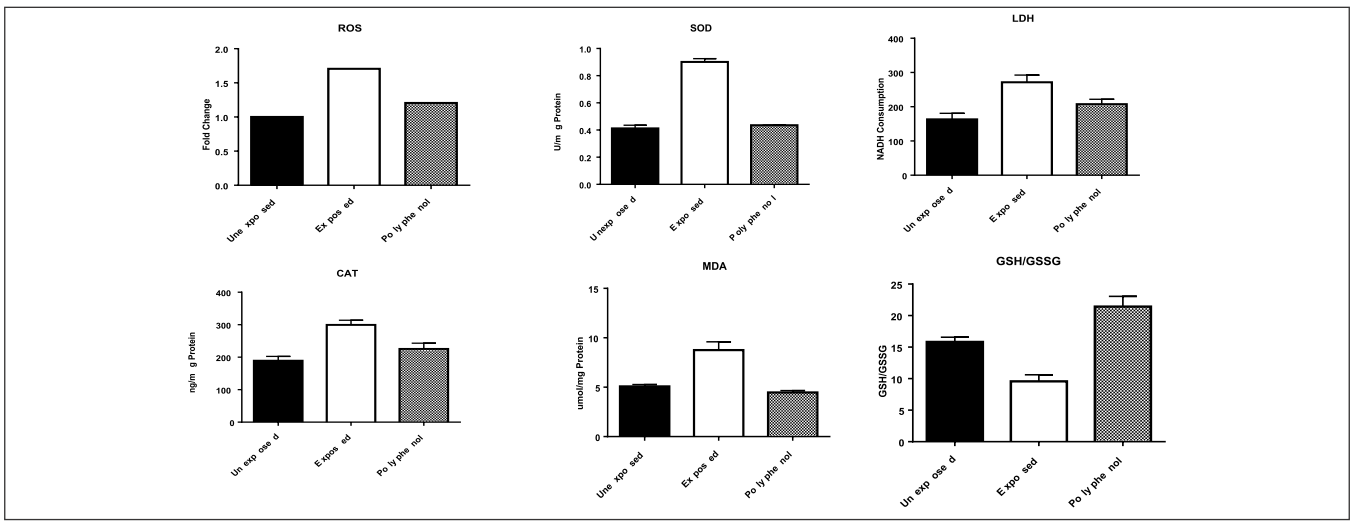


Figure 8: (A-F)Effect of polyphenols fraction supplementation on antioxidative stress parameters in Blood.

that enhanced by 58% in exposed control rats as compared to unexposed control, reduced by 25% in both the cases in comparison to exposed control. The LDH level in exposed control rats increased by 66% in comparison to unexposed control whereas the reduction as compared to control exposed for SBT-5 was significant 24%. Much protective effect against generation of MDA was seen as PRF significantly reduced the levels by 49% comparison to exposed control rats. The mean ratio of GSH/GSSG was significantly decreased during CHR exposure (40%) in comparison to unexposed control. Treatment with PRF restored the GSH/GSSG ratio.The trend of antioxidative and stress parameters in case of muscles is given in Fig.9 (a-f).

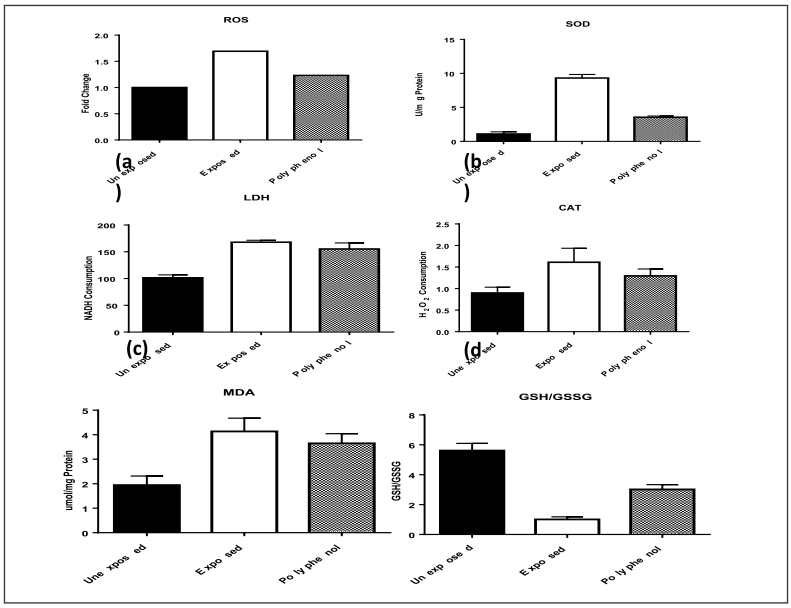


Figure 9 (a-f):Effect of Polyphenolic fraction supplementation on antioxidative stress parameters in Muscle.

Exposure to CHR led to an enhanced oxidative stress as evident by increased ROS, SOD and CAT levels in the exposed control rats. Further supplementation with PRF significantly ameliorated the oxidative stress. The SOD levels that were increased in exposed control rats as compared to unexposed control significantly reduced by 62% after administration of the fraction as compared to exposed control. The levels of CAT that were increased by 79% after exposure in control rats reduced by 20% after the administration of the fraction. CHR induced increase (67%) in LDH levels in muscles sample were lowered significantly by the fraction (24% decrease). Further the enhanced MDA levels during CHR exposure were decreased non-significantly by 12%.Fig.10 (a-f) shows results on antioxidant status and stress markers in the heart samples.

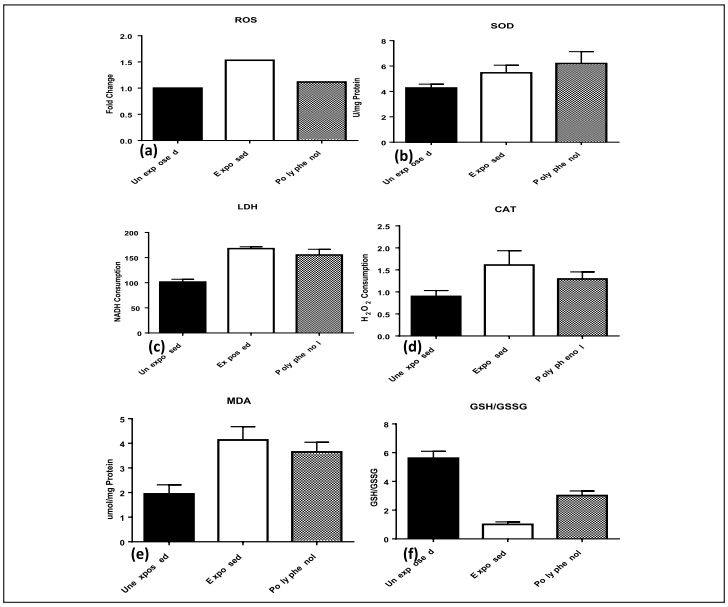


Figure 10 (a-f):Effect of Polyphenolic fraction supplementation on antioxidative stress parameters in Heart.

Heart samples when analysed for the generation of ROS under multiple stress showed 53% increase in control exposed rats as compared to unexposed control. The supplementation of PRF reduced the generation of reactive oxygen (ROS) by 12% as compared to exposed control. There was an increase of 45% in the levels of Superoxide dismutase (SOD) on attaining T_{rec}23°C in exposed control rats versus unexposed control. This increase reduced by 12% after administration with PRF. In the fraction treated rats the value of CAT that increased by 109% in exposed control rats reduced significantly by 43%. In case of LDH the observed increase of significant 52% in exposed control rats in comparison to unexposed control decreased by 19% after administration with PRF. The MDA level in exposed control rats increased by 120% in comparison to unexposed control whereas the reduction as compared to control exposed for the fraction was only 3%. There was 12% decrease in the ratio of GSH/GSSG which improved by 79 % as compared to exposed control rats after oral supplementation of the fraction.

Immune response generated by fractions

The initiation of immune response generated by the fractions, SE, PM and SM was measured through indirect Elisa. The result indicates that SM is showing 74% increment in TT specific wIgG titers whereas SE showed only 60% and PM showed 40% augmentation when compared with TT alone group. Similarly, when compared the immunogenic capabilities of SM, SE and PM with Ova, it was observed that SM exhibited 176%, SE showed 60.5% and PM revealed 67.6% enrichment in Ova specific immune response generated by the fractions when compared with Ova alone group. The fractions alone did not generate any significant immune response. There was approximately 3% increment in the immune response generated by the fractions (Table 1) (Singh et al. 2017).

Table-2: Comparison of immune response of different fractions of SBT-5 with Ova and TT antigens.

Name of Fraction	Alone	Ova	TT
	-	1 ± 0.02	1 ± 0.019
Successive Methanol (SM)	2.65 ± 0.09	176.47 ± 43.23	74.66 ± 21.45
Parallel Methanol (PM)	00 ± 0.00	67.61 ± 8.75	40.67 ± 8.67
Successive Ethyl acetate (SE)	2.5 ± 0.06	60.56 ± 9.67	60.47 ± 14.67

Discussion

Modern analytical techniques have provided opportunity for the researchers to identify and separate active plant components which are particularly of some importance. Although the nature of plant components is very complicated but it has never deterred the quest to classify active constituents of herbal extracts. Our target was very specific. We have already established the antioxidant and adaptogenic potential of SBT-5 (Sharma et al. 2015).Our aim was to identify its active fraction.

HPTLC is a suitable method for estimation of chemical constituents present in plants materialswith minimum sample clean up requirement (Patil et al. 2012 ; Rakesh et al.2009).HPTLC fingerprinting identifies 3 proteins and 2 polyphenols in SM, the fraction that has shown better adaptogenic potential as compared to SE and PM at a four times lower dose.

SM at a dose of 1 µg/animal shows significantwIgG titers with the strong antigen TT (Gupta and Siber 1994) as well as weak antigen Ova, when tested *in vivo*. Ovais the major protein constituent of chicken egg whites (Sun, 2006). It is widely used as an antigen for immunization research. Enhancement of Ova specific wIgG by SM indicates remarkable potential of this fraction. TT generated immune response is also heightened by SM. Therefore, the immunomodulatory activity of SM fraction derived from Seabuckthorn leaves demonstrates better outcomes with TT and Ova at a very low dose. This also indicates that the extracts and its major constituents have the potential to be developed into agents for the treatment of a variety of disorders including inflammation (Tanwar et al. 2018).

Both SM and PRF show comparable adaptogenic potentials and later also significantly reduce the recovery time from hypothermia. Thus, phenols might be playing significant role in recovery from CHR generated multiple stresses. Recently polyphenols have been associated with anti-inflammatory, antioxidant, immunomodulatory, and apoptotic properties (Kaulmann et al. 2016). The dietary intake of polyphenols could be as high as 1 g/day, which is much higher than that of all other classes of phytochemicals and known dietary antioxidants (Manach C, 2004; Scalbert and Williamson 2000; Wisnuwardani et al. 2019). Polyphenols-rich plants and their extracts ameliorate the severity and progression of Inflammatory Bowel Disease (Michielan andD'Incà2015; Rahman et al. 2018).Previous study also proved potent antioxidant and antibacterial properties of polyphenolic rich compounds(Yogendra et al. 2013; Bouarab-Chibaneet al. 2019). These compounds could also prevent oxidative damage in the liver (Maheshwari et al. 2011). Phenolic compounds such as flavonoids, phenolic acids, diterpenes

and tannins have received attention for their high antioxidative activity (Bertelli et al. 2015; Dibanda et al. 2020). Bioassay-guided fractionation of extracts obtained from one of the other high altitude plant *Rhodiola* has shown that the active components are mainly phenylethanolic derivatives and phenylpropanoids (Sharma and Misra, 2018). Flavonoid rich fraction of HR was found to be safe and effective for radio protection (Chawla et al. 2007). Our own studied also demonstrated significant amounts of total phenol and flavonoids content in SBT-5 with identification and quantification of Gallic acid which is a potent antioxidant (Sharma et al. 2015).

Thus, it could also be suggested that the polyphenols contributes significantly towards the anti-stress adaptogenic potential of SBT-5, SM and PRFwhen studied using CHR animal model.Under multiple stressful conditions of CHR oxidative stress is generated. This study incorporates those parameters that give us a purview of oxidative damage, like ROS for oxidative stress, MDA, a marker for lipid peroxidation and LDH, a marker of stress induced membrane damage and important antioxidants like SOD, CAT and GSH. PRF possesses great potential in terms of oxidative stress protection than the crude extract as a whole because it had shown comparable results at four times lower dose. This may be attributed to the fact that flavonoids can directly scavenge molecular species of active oxygen: $^{\cdot}O_2$-*superoxide*, H_2O_2 - *hydrogen peroxide*, OH-*hydroxyl radical*, ^{1}O-*singlet oxygen* or *peroxyl radical*. Their antioxidant action resides mainly in their ability to donate electrons or hydrogen atoms (Borges Bubols et al. 2013; Sarian and Williamson 2017).

Conclusion

SM has shown its potential both as an adaptogen as well as immunomodulator. Its adaptogenic potential is comparable to PRF but both fractions are better than SBT-5 as they have shown their activities at four times lower dose. In addition, Polyphenols fraction also fastens recovery from CHR induced hypothermia.This study gives us a lead that PRF plays important role in adaptogenic and antioxidant properties.

Conflict of Interest

The authors declare that they have no conflicts of interest concerning this article.

Acknowledgements

We are grateful to Dr. Srinivasan Narsimhan from Asthagiri Herbal Research Foundation, Chennai, Tamil Nadu, India for help rendered in preparing SBT fractions. This study was supported and funded by the Defence Research and Development Organization, Ministry of Defence, Government of India. We are especially thankful to Director, DIPAS for constant support and encouragement.

References

1. **Aggrawal A, Tewari S, Shivakumar S, Ilango K, Singh RG, Dubey GP** (2014) In: Seabuckthorn (Hippophae L.): A multipurpose wonder plant (Ed: Virendra Singh). New Delhi, India. 4:465-78.
2. **Bertelli D, Maietti A, Papotti G, Tedeschi P, Bonetti G, Graziosi R, Brandolini V, Plessi M** (2015) Antioxidant activity, phenolic compounds, and NMR characterization of balsamic and traditional balsamic vinegar of Modena. *Food Anal methods*. 8:371-379.
3. **Borges Bubols G, da Rocha Vianna D, Medina-Remon A, von Poser G, Maria Lamuela-Raventos R, Lucia Eifler-Lima V, Cristina Garcia S** (2013) The antioxidant activity of coumarins and flavonoids. Mini Rev Med Chem.13:318-34.
4. **Bouarab-Chibane L, Forquet V, Lantéri P, Clément Y, Léonard-Akkari L, Oulahal N, Degraeve P, Bordes C** (2019) Antibacterial properties of polyphenols: characterization and QSAR (Quantitative structure–activity relationship) models. FrontMicrobiol.https://doi.org/10.3389/fmicb.2019.00829.
5. **Chawla R, Arora R, Singh S, Sagar RK, Sharma RK, Kumar R, Sharma A, Gupta ML, Singh S, Prasad J, Khan HA, Swaroop A, Sinha AK, Gupta AK, Tripathi RP, Ahuja PS** (2007) Radioprotective and antioxidant activity of fractionated extracts of berries of Hippophaerhamnoides. *J Med Food*. 10: 101-09.
6. **Dibanda RF, Akdowa EP, Tongwa QM** (2020) Effect of microwave blanching on antioxidant activity, phenolic compounds and browning behaviour of some fruit peelings. Food Chem..https://doi.org/10.1016/j.foodchem. 2019.125308.
7. **Geetha S, SaiRam M, Mongia SS, Singh V, Ilvazhagan G, Sawhney RC** (2003) Evalation of antioxidant activity of leaf extract of seabuckthorn (Hippophaerhamnoides L.) on chromium (VI) induced oxidative stress in albino rats. *J Ethnopharmacol* 87:247-251.
8. **Gupta A, Kumar R, Pal, K, Banerjee PK, Sawhney RC** (2005) A preclinical study of the effects of seabuckthorn (Hippophaerhamnoides L.) leaf extract on cutaneous wound healing in albino rats. *Int J. Low Extrem Wounds*. 4:88-92.
9. **Gupta RK, Siber GR** (1994) Comparative analysis of tetanus antitoxin titers of sera from immunized mice and guinea pigs determined by toxin neutralization test and enzyme-linked immunosorbent assay. Biologicals 22:215-219.
10. **Kaulmann A, André CM, Schneider YJ, Hoffmann L, Bohn T** (2016) Carotenoid and polyphenol bioaccessibility and cellular uptake from plum and cabbage varieties. *Food Chem* 197:325-332.
11. **Maheshwari DT, Yogendra Kumar MS, Verma SK, Singh VK, Singh SN** (2011) Antioxidant and hepatoprotective activities of phenolic rich fraction of Seabuckthorn (*Hippophaerhamnoides* L.) leaves. Food ChemToxicol 49:2422-2428.

12. **Manach C, Scalbert A, Morand C, Rémésy C, Jimenez L** (2004) Polyphenols: food sources and bioavailability. Am J ClinNutr 79:727– 747.

13. **Michielan A, D'Incà R** (2015) Intestinal permeability in inflammatory bowel disease: pathogenesis, clinical evaluation, and therapy of leaky gut. Mediators inflamm. https://doi.org/10.1155/2015/628157

14. **Mohammad Azmin SN, Abdul Manan Z, Wan Alwi SR, Chua LS, Mustaffa AA, Yunus NA** (2016) Herbal processing and extraction technologies. Sep Purif Rev 45:305-320.

15. **Olas B, Skalski B, Ulanowska K** (2018) The Anticancer Activity of Sea Buckthorn [*Elaeagnusrhamnoides* (L.) A. Nelson]. Front Pharmacol. https://doi.org/10.3389/fphar.2018.00232.

16. **Patil VP, Kurhade SD, Devdhe SJ, Kale SH, Wakte PS** (2012) Determination of Gallic Acid by HPTLC as Quality Control Parameter in Herbal Formulation: *Triphalachurna.* Int J ChemAnalSci 3: 1546-1549.

17. **Rahman SU, Li Y, Huang Y, Zhu L, Feng S, Wu J, Wang X** (2018) Treatment of inflammatory bowel disease via green tea polyphenols: possible application and protective approaches. Inflammopharmacology 26:319-30.

18. **Rakesh SU, Salunkhe VR, Dhabale PN, Zburade KB** (2009) HPTLC method for quantitative determination of gallic acid in hydroalcoholic extract of dried flowers of *Nymphaea stellata* Willd. Asian J Res Chem 2:131-134.

19. **Ramachandran U, Divekar HM, Grover SK, Srivastava KK** (1990) New experimental model for the evaluation of adaptogenic products. *J Ethnopharmacol.* 29:275-281.

20. **Roy M, Datta A** (2019) Fundamentals of Phytochemicals. In: Cancer Genetics and Therapeutics. Springer, Singapore. https://doi.org/10.1007/978-981-13-9471-3_3.

21. **Roy P, Mandal P, Panda S, Roy SM, Subba A** (2018) Pharmacognosy and phytochemical screening of some plant derived medicine to treat dysmenorrheal pain by the Rajbanshi Community. *Pharmacogn J.* 10 :738-746.

22. **Saggu S, Gupta V, Sawhney RC, Banerjee PK, Kumar R** (2006) Analysis of heavy metal in herbal extracts of high-altitude growing plants Hippophaerhamnoides and *Rhodiola imbricata*. Toxicol Int13: 111–117.

23. **Sarian MN, Ahmed QU, Mat So'ad SZ, Alhassan AM, Murugesu S, Perumal V, Syed Mohamad SN, Khatib A, Latip J** (2017) Antioxidant and antidiabetic effects of flavonoids: A structure-activity relationship-based study. Bio Med Res Int. https://doi.org/10.1155/2017/8386065.

24. **Scalbert A, Williamson G.** (2000) Dietary intake and bioavailability of polyphenols. J Nutr130:2073S–2085S.

25. **Sharma P, Kirar V, Meena DK, Suryakumar G, Misra K** (2012)Adaptogenic activity of Valerianawallichii using cold, hypoxia and restraint multiple stress animal model. Biomed Aging Pathol2:198–205.

26. **Sharma P, Kumar R, Prasad R, Suryakumar G** (2014) Anti-stress and Adaptogenic Potentials of Seabuckthorn (*Hippophaerhamnoides* L.). In: Seabuckthorn (Hippophae L.): A multipurpose wonder plant (Ed.:Virendra Singh). New Delhi, India, pp 418-426.

27. **Sharma P, Suryakumar G, Singh V, Misra K, Singh SB** (2015) In vitro antioxidant profiling of seabuckthorn varieties and their adaptogenic response to high altitude-induced stress. *Int j Biometeorol.* 59:1115-1126.

28. **Sharma P, Misra K** (2018) *Rhodiola* sp.: The Herbal Remedy for High-Altitude Problems. In: Misra K, Sharma P, Bhardwaj A (eds) Management of High Altitude Pathophysiology, 1st edn. Elsevier, London, pp-81-90.

29. **Singh AK, Paul AD, Tanveer N** (2013) Bioactivity guided extraction of Seabuckthorn (Hippophaerhamnoides L ssp. Turkestanica) leaves. J Sci Indus Res 72:307-311.

30. **Singh D, Tanwar H, Jayashankar B, Sharma J, Murthy S, Chanda S, Singh SB, Ganju L** (2017) Quercetinexhibits adjuvant activity by enhancing Th2 immune response in ovalbuminimmunizedmice. BiomPharmaco 90:354-360.

31. **Sun HX** (2006) Adjuvant effect of Achyranthes bidentata saponins on specific antibody and cellular response to ovalbumin in mice. Vaccine 24:3432-3439.

32. **Tanwar H, Singh D, Singh SB, Ganju L** (2018) Anti-inflammatory activity of the functional groups present in Hippophaerhamnoides (Seabuckthorn) leaf extract. Inflammopharmacology 26:291-301.

33. **Tanwar H, Chanda S, Agrawal MJ** (2019) Immunopotentiating activity of an herbal adjuvant: its benefits thereof. Int J Complement Alt Med 12:122-124.

34. **Wisnuwardani RW, De Henauw S, Androustsos O** *et al* (2019) Estimated dietary intake of polyphenols in European adolescents: the HELENA study. Eur J Nutr 58:2345-2363.

35. **Yogendra KMS, Tirpude RJ, Maheshwari DT, Bansal A, Misra K** (2013) Antioxidant and antimicrobial properties of phenolic rich fraction of Seabuckthorn (Hippophaerhamnoides L.) leaves in vitro. Food Chem 141:3443-3450.

SAVE THE ENVIRONMENT (STE) was founded and registered on 19th November 1990. In 1992 with the collaboration of WWF (India), the organization started working to combat arsenic poisoning problem of water in the arsenic prone areas of West Bengal. Since then STE has been involved in various projects related to combat arsenic problem in India.

Our Vision
To protect present and future generations from various environmental hazards.

Our Mission
To create awareness and motivation among rural communities & provide cost effective, energy efficient & environment friendly technologies.

Our Activities
Conducting interactive sessions, workshops/ seminars, awareness programs, field operations through projects, science fairs, posters & quiz competitions.

Please join us and become part of our family
by enrolling yourself as Life Member of STE Family

Mail us at
info@stenvironment.org
save1990env@yahoo.co.in

Know about us at
www.stenvironment.org