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Article *in* International Research Journal of Pure and Applied Chemistry · October 2020

DOI: 10.9734/IRJPAC/2020/v21i1730261

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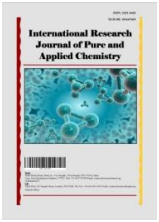

International Research Journal of Pure & Applied Chemistry

21(17): 10-17, 2020; Article no.IRJPAC.61303
ISSN: 2231-3443, NLM ID: 101647669

Proximate Analysis and Evolution of Energy Value from Leaves and Stem of Sword Fern: *Nephrolepis exaltata*

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Authors' contributions

This work was carried out in collaboration among all authors. Author DKS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors RSD and KRS managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IRJPAC/2020/v21i1730261
Editor(s):
(1) Dr. Hao-Yang Wang, Shanghai Institute of Organic Chemistry, China.
Reviewers:
(1) Ayobami Omozemoje Aigberua, Niger Delta University, Nigeria.
(2) Syed Mubashar Sabir, University of Poonch Rawalakot, Pakistan.
Complete Peer review History: http://www.sdiarticle4.com/review-history/61303

Original Research Article

Received 15 July 2020
Accepted 22 September 2020
Published 05 October 2020

ABSTRACT

The purpose of present study was to prognosticate the amount of each nutrient in the Arial parts of sword fern "*Nephrolepis exaltata*". The study was conducted at Sarva Vidhyalaya Campus, Kadi, Gujarat (India) during the period of December-2019 to March-2020. The prognostication of nutritive component including ash%, moisture%, Crude Protein%, Crude Fat%, Crude fibers% and Carbohydrate was taken from AOAC and Standard Lowry method for estimation of protein. The proximate analysis indicates the monumental presence of crude protein and carbohydrates, moderate presence of crude lipid and fibers. The ash content was 8.65% and 12.42% in leaves and stem respectively, which manifests the existence of inorganic minerals. The Crude protein was recorded as 21.25% and 30.0% in leaves and stems respectively, whereas the crude lipid was 8.41% and 1.47% respectively. The leaves and stems were found to be a good source of energy value with 359.766 Kcal/100 gm and 313.266 Kcal/100 gm respectively.

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Keywords: Nephrolepis exaltata; proximate analysis; Lowry method; energy value; AOAC (Association of Official Analytical Chemists); pteridophyte; vascular plants.

1. INTRODUCTION

In 1860, Henneberg and Stohmann developed a method for quantitative analysis for discriminate the different macronutrients in feed, was explored as proximate analysis. Proximate analysis is not wholly nutrient analysis, rather it is a partitioning of both nutrients and non-nutrients into categories based on common chemical properties. In Proximate Analysis, based on the chemical properties, the compounds in a feed are partitioned into six different categories. They include: moisture, ash, crude protein, crude lipid, crude fibre, nitrogen-free extracts (digestible carbohydrates) [1].

To acquire the approximate amounts of substances within a material the Proximate analysis can be the best scientific inquiry. This method is traditionally utilized in contrasting scientific fields to study diverse materials such as animal feed, coal, and bio-fuels etc. The process is quite intricate involving extraction of one material through miscellaneous solvents or extraction of different materials through single solvent. The hazard potential of chemicals can be determined by proximate analysis. This information can be used to create quality controls for various materials which ensure that they are healthy enough to be consumed by humans or animals [2].

Proximate analysis of plant is the best way to ensure the composition of essential biomolecules such as protein, fats, and carbohydrates etc. In human food products, nutrient levels such as proteins, fats and carbohydrates are often determined [3].

Desert area is the most engrossing part of the mother earth and the point of origin for many plants' species, from ferns to modern dicots. Pteridophytes including ferns and fern-allies are non-flowering, vascular and spore bearing plants. They are ostentatious plants of the earth's vegetation and from evolutionary perspective they are important as they show the evolution of vascular system and exposure of seed habit in the plants. They are abundant in moist tropical and temperate forests and grow in different intermediate regions from sea level to the highest mountains [4]. Ferns are vascular plants that do not produce seeds. *Nephrolepis exaltata*, an epiphytic and terrestrial fern was taken for current study. There are around thirty species in the genus *Nephrolepis* [5]. *Nephrolepis exaltata* is a customarily found pteridophyte and vocalized as "Boston fern" or "sword fern" which is classified in the family *Nephrolepidaceae* [6].

Nephrolepis is easily elucidated and morphologically quite sui generis plant; the stem is a minuscule erect stock with closely tasseled fronds. It has a willowy lateral branch bearing numerous roots and occasional buds which develops into new plants. The genus *Nephrolepis* belongs to Family Nephrolepidaceae, which includes 35 Genera and 303 accepted taxa overall with 2 Subspecies. The order of plant is 'Polypodiales' [7]. The plants of this order are also known for their resistivity in desert area.

Different studies have established the presence of antioxidant activity and also anti-inflammatory property in many species of *Nephrolepis* [8]. Some studies on mice shows that the extract preparation of *Nephrolepis biserrata* given at 100 mg/kg within an hour interval can reduce the level of inflammation. The leaves of *Nephrolepis biserrata* are used for treatment of blisters, boils, abscesses, sores, abdominal pains, wounds and cuts [9].

The result of the proximate composition for some plants showed that the seed oil possessed good physicochemical properties which are the main component of edible oil. As such, this type of plant parts could be utilized successfully as a source of edible oil for human consumption and for industrial applications [10].

Despite the progress made in research of *Nephrolepis exaltata* during the past decades, some phytochemical constituents of the plants are yet to be discovered. As such, the plant yet retains further potential to provide the master stroke for resolving some of the globe's major health problems. However, this study seeks to examine the potential of *Nephrolepis exaltata* for nutritional use. Although some studies revealed that the aqueous extract of fern *Nephrolepis exaltata* may be cardiotoxic to some Arthropods like cockroaches in a dose-dependent manner. Some of unidentified terpene found in aqueous extract of *Nephrolepis exaltata* could play a part for the cardiotoxic effect of in cockroaches [11]. Some studies revealed facts about the allergic effects of Boston fern *Nephrolepis exaltata*,

which is commonly vocalized as 'Bostoniensis' [12]. Proximate analysis is most dependable strategy to find nutritional composition of numerous plants and their parts. Some proximate analysis methods have been used to interpret the nutrition relationship between monocots Maize and sorghum [13].

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

The fresh plants were collected from a local surrounding in Kadi (23.2973°N, 72.3302°E) during the month of December 2019. Identification and authentication of the plants was done by Taxonomist and Associate Professor, Dr. K.J Bhatt at the Department of Biology, Pramukh Swami Science and H.D Patel Arts College, Kadi.

2.2 Processing of Plant Materials

Nephrolepis exaltata plant leaves and stem were air dried at 28°C to 40°C for 30 days. Thereafter, samples were milled into fine powder using electric blender. This was done in order to increase the surface area of sample for improved solvent penetration of cells and enhanced extraction of secondary metabolites.

Although fresh and dried samples can be applied in plant extraction studies, dried samples are mostly preferred when considering the time needed for experimental design. Vongsak compared the extraction of fresh and dried plant parts and concluded that fresh samples are fragile, with tendency to deteriorate faster than dried samples, hence extraction is faster in dried samples [14].

2.3 Proximate Analysis

The AOAC methods were used to determine proximate analysis of all plant samples. Powdered plant material was used for analysis of % Ash, % moisture, Crude protein, Crude lipid, Carbohydrates and elemental composition of the selected plant species.

The Proximate analysis was carried out by following scheme-

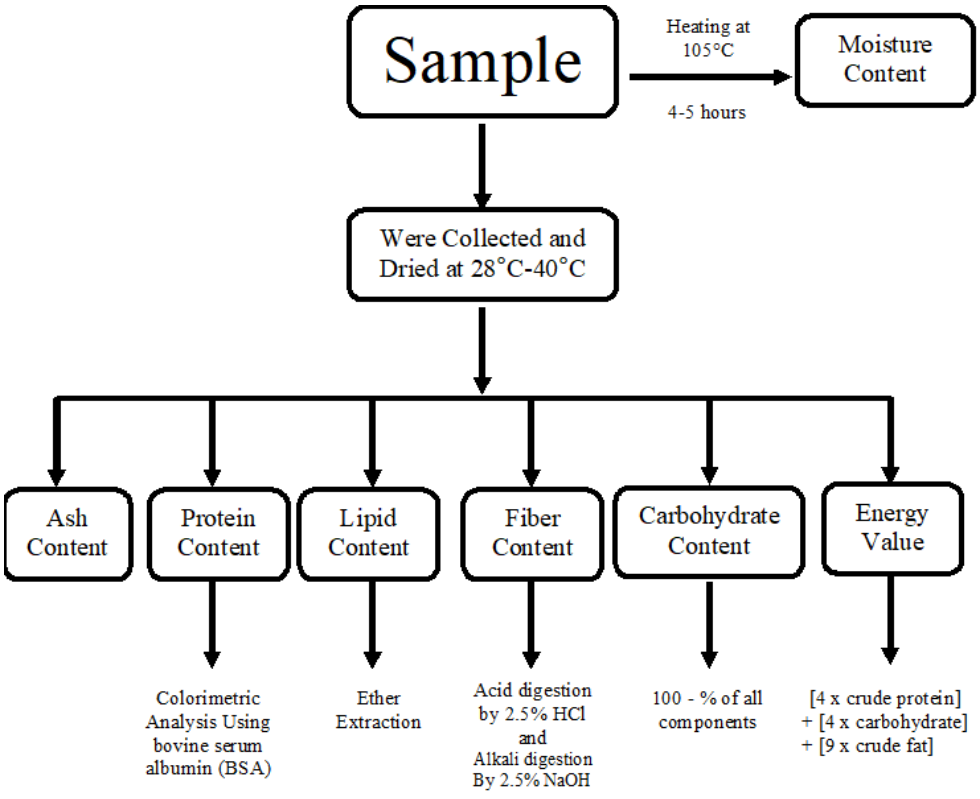

Fig. 1. Scheme for proximate analysis of *Nephrolepis exaltata*

2.3.1 Determination of % ash

Ash was determined according to AOAC (2000) method. At 660°C a clean crucible was heated in a muffle furnace for an hour, then in a desiccator it was cooled and weighed as (W_1). For the determination of ash content, 10 g dry sample was taken in the crucible. The sample was scorched over the burner with the help a blowpipe. Thereafter, the crucible containing sample was heated at 550°C for 6-8 hours in a muffle furnace. When6 the embers left in crucible after the complete ignition the furnace was turnoff. After cooling the crucible at room temperature, it was weighed as (W_2). Percent ash was calculated as follow.

$$\%\text{Ash} = \frac{Wt.of\ Ash\ (W_2 - W_1)}{wt.of\ sample} \times 100$$

2.3.2 Determination of the moisture

Standard AOAC method was followed to deduce the Moisture contents. A dry empty pre-weight (W_1) clean petri-dish (with lid) was filled with 1 gram of sample. Samples were oven-dried at 105°C for 4-5 hours until constant weight was obtained. After which Sample was removed and placed in desiccator for 30 minutes in order to cool it. After cooling the dish, the final weight (W_2) was measured was weighted. Percent was calculated as follows:

$$\%\text{Moisture} = \frac{Wt.of\ residue\ (W_2 - W_1)}{wt.of\ sample} \times 100$$

2.3.3 Determination of proteins

The content of proteins can be ascertained using different methods. From a comparative analysis to determine total protein using the Kjeldahl method and several spectrophotometric methods in a number of different samples, it was concluded that the Lowry method showed the lowest variance using BSA (Bovine Serum Albumin) as standard protein for precise absorption [15].

Due to simplicity and availability of reagents The Lowry method has been widely used for protein determination. However, besides aromatic amino acids, a wide range of other compounds react with the Folin–Ciocalteu reagent [16]. The modified Lowry protein measurement was conducted according to the method described by Hartree in 1972 [17].

2.3.3.1 Proteins extraction

For the estimation of protein in different plant parts, samples were homogenized separately. About 10% trichloroacetic acid (TCA) was applied to the plants since TCA has the property to dissolve macromolecules such as proteins, DNA, and RNA so it is widely used in biochemistry for the precipitation. After preparation of homogenized solution, centrifugation followed at 5000 rpm for 10 minutes. The Supernatant liquid portion was discarded and pellets containing the protein were recovered. Pellets were again suspended in 5 ml of 10% cold TCA and recentrifuged for 10 minutes. Supernatant was again discarded and the precipitate was dissolved in 10 ml of 0.1 N NaOH. The solution was used for protein estimation.

2.3.3.2 Quantitative estimation of proteins

Using the protocol of Lowry *et al.*, 1951, total protein content was estimated in about 1 ml extract.

1. For preparation of a stock solution of bovine serum albumin (1 mg/ml), 1 N NaOH was used.
2. The five-working solutions of different concentrations (2, 4, 6, 8 and 10 ml) from the working standard solution were taken in series of test tubes.
3. In another set of test tubes 0.1 ml and 0.2 ml of the sample extracts were taken and the volume was made up to 1 ml in all the test tubes using distilled water.
4. A mixture solution (A) was prepared by mixing 50 ml of 2% Na_2CO_3 in 0.1 N NaOH and 1 ml of 0.5% $CuSO_4$. $5H_2O$ in 1% sodium potassium Tartrate.
5. The 5 ml prepared mixture (A) was added to each test sample at room temperature.
6. The prepared test samples were left undisturbed for a period of 10 min.
7. Subsequently, A mixture of 0.5 ml of distilled water mixed with 0.5 ml FC reagent B was added to each mixture tube at room temperature (25°C) for 30 minutes until the blue color developed.
8. The Equip-Tronics colorimeter (µP based Inbuilt Filter-Model No-WQ-652) was adjusted at wavelength of 720 nm and set at 100% transmittance using blank before taking the readings of the standard and the test samples respectively.
9. Five replicates were examined in each case and their mean values were recorded.
10. A regression curve was worked out of various concentrations of the standard

solutions against their respective absorbance in accordance to the Beer's law.

2.3.4 Determination of crude lipid (fat content)

There are a variety of test methods for fats, but generally the method can be broken down into "crude" or total fats. "Crude" methods involve dissolving the material in a solvent such as ether or hexane followed by solvent evaporation. The material that remains is called "crude fat". In this case, ether was used as solvent to dissolve the lipid content.

1. A thimble with dry sample was prepared and its weight recorded as W_1.
2. The extraction was done using Soxhlet extractor with diethyl ether as solvent.
3. The thimble was placed into a thimble holder and the holder was clipped.
4. Then after a sufficient amount (one glass reclaiming tube full - approximately 40 ml) of diethyl ether was poured into RBF (Round bottom flask) of sohxlet apparatus, so the plant material in the thimble could be extracted.
5. The RBF was coupled to the extractor with the ring clamp tightly adjusted.
6. The temperature was set to 40^{o}C in heating element.
7. The heater switch, the main power switch, and the condenser water were turned on.
8. As the ether solvent boiled, glassware was inspected for leaks so as to ensure that there were no volumetric losses.
9. Extraction was carried out using standard methods for 16 hours at Low setting (condensation rate of 2 to 3 drops per sec).
10. After extraction, the temperature of heating element was lowered down by shutting down the power and water supply and allowed the ether to drain out of the thimbles (about 30 min).
11. Remove the thimble from the holder, and rinse the holder with small portions of diethyl ether from the wash bottle.
12. The extract was transferred into a pre-weighed beaker (W_2) for further evaporation at room temperature.
13. Room temperature drying was employed in order to avoid possible explosion from oven-induced drying of the ether solvent.
14. The weight of beaker and residue (W_3) was recorded on completion of the drying process.

    Note: Excessive drying may oxidize the fat and give high results.
15. The remaining residue in thimble was used for fiber analysis.

$$\%\text{Crude Lipid} = \frac{Wt.of\ extract\ (W_3 - W_2)}{wt.of\ sample\ (W_1)} \times 100$$

2.3.5 Determination of crude fibers

Crude fiber was determined by acid and alkali digestion method using fiber tec apparatus by following AOAC (2000) [18].

1. The weighed (W1) thimble residue samples were first digested with acid and then with alkali.
2. The sample was transferred into a clean glass beaker a clear beaker and 100 ml of 2.5% HCl was added to it.
3. The mixture was boiled with stirring for about half an hour. It was then drained into the beaker.
4. The fiber residue was again digested in 2.5% NaOH, similar to the acid digestion process.
5. The residue collected was transferred to a pre-weighted (W_2) dried crucible to remove the moisture.
6. The crucible was then kept in furnace for red dull heat till the formation of white and grey ash. The crucible was cooled in desiccators and weighed again. (W_3)
7. The loss in weight of the dry residue upon ignition was taken as the amount of crude fiber. Percent crude fiber was calculated as follows-

$$\%\text{Crude Fiber} = \frac{Wt.of\ dry\ residue\ (W_3 - W_2)}{wt.of\ sample\ (W_1)} \times F \times 100$$

Where,

F = Value of crude fat, W_1 = Weight of Sample, W_2 = Weight of dry crucible, W_3 = Weight of crucible after heating

2.3.6 Determination of carbohydrate contents

Carbohydrates were determined by subtracting the weights of protein fats, crude fibers, ash, and moisture contents from 100.

$$\text{TCH (\%)} = 100 - \%(\text{CP} + \text{A} + \text{CF} + \text{M})$$

2.3.7 Determination of energy value

Carbohydrates give average gross energy values of 4.2 kcal or 17.6 kJ per gram, fat gives 9.4 kcal, or 39.4 kJ per gram and protein gives 5.65 kcal or 23.7 kJ per gram. So, the energy values were

estimated by calculation method using the formula [19],

Energy value (g/100g) = [5.65 x crude protein] + [4.2 x carbohydrate] + [9.4 x crude fat]

3. RESULTS AND DISCUSSION

Data in Table 1 represents the "Proximate Analysis and Energy Value" of *Nephrolepis exaltata*. The dry sample of leaves was light green in color with specific agreeable odor whereas the dry sample of stem was subtle colored. The percentage yield of Ash content was found to be higher in stem, which indicates the presence of high mineral content. The moisture content in leaves and stem was found to be very high, ranging between 21.78% in leaves and 24.83% in stem. Apart from moisture the major chemical constituent found was carbohydrate, recording a maximum content of maximum content 38.32% in leaves and 30.94% in Stem. Crude protein content was estimated as 21.25% in leaves and 30.00% in stem, while crude fat (Lipid) content observed was 8.41% in leaves and 1.47% in stem. The crude fiber content with maximum concentration 1.59% was determined in leaves while minimum amount 0.34% was found in stem.

Table 1. Proximate analysis and energy value of *Nephrolepis exaltata* plant leaves and stem (% of dry samples)

Composition	*Nephrolepis exaltata* (Leaves)	*Nephrolepis exaltata* (Stem)
Ash %	8.65%	12.42%
Moisture %	21.78%	24.83%
Crude Protein %	21.25%	30.00%
Crude Lipid %	8.41%	1.47%
Crude Fiber %	1.59%	0.34%
Carbohydrate %	38.32%	30.94%
Energy Value (Kcal/100 gm)	359.766	313.266

The moisture content was observed 21.78% in leaves and 24.83% in stem respectively; The result was compared favorably with that of Oloyede *et al.* (2012) and Adebiyi el at. (2016) who worked on the *Nephrolepis furcans* and *Nephrolepis cordifolia* (L) in Nigeria respectively [20,21]. This indicates that the amount of moisture varies from species to species and from geographical locations. Low moisture content promotes shelf life of plant and hamper the growth of microorganism. The ash content gives an indication regarding the amount of minerals present in a particular sample, which are important in many biochemical reactions functioning as co-enzyme and aid physiological functioning of major metabolic processes in the body. It contains inorganic material of the plant because ashing destroys all the organic material present in the sample. High ash content in any food substance is an indication of high mineral content [22].

In contrast with other species, *Nephrolepis exaltata* do have higher protein level. The results of the protein analysis observed in this study; is almost similar what was previously reported for *Nephrolepis exaltata* by Johnson M *el at.* (2016). The relative positions of the protein bands were studied for *Nephrolepis exaltata* (L.) through SDS-PAGE and the plant showed maximum number of protein bands [23]. The Fiber content was found in small extent as it was observed in *Nephrolepis furcans* by Oloyede *et at.* 2012, which revealed the lower presence of Fat-free organic substances [20]. The result of the carbohydrate analyses of the plant is reported higher than the other species of plant which shows the food storage capability.

From the above results, it was shown that *Nephrolepis exaltata* has lesser amount of nutrients such as carbohydrates and crude fiber, but it had been found major amount of carbohydrate and protein in it, that determine nutritional value of the fern.

4. CONCLUSION

In conclusion the proximate analysis of *Nephrolepis exaltata* showed that the Sword fern is a good source of essential nutrients like protein, carbohydrate, fat, fibers. Protein analysis by Lowry method indicated that sword fern might be a rich source of isolatable protein. The plant has high ash content, thereby indicating the presence of minerals and Inorganic components. It can be inferred that due to the presence of primary constituents the several solvent extracts from Sword fern can be utilized in different sectors. The presence of different phytochemicals, anti-microbial, anti-fungal potential can also be stabilized. This study suggests that *Nephrolepis exaltata* can be a promising material for pharmaceutical application.

ACKNOWLEDGEMENTS

We thank Dr. A. S. Gor, Principal, PSSHDA and Director, HVPG, kadi and Dr. Minal Trivedi, Principal, HVPGR, Kadi for their support and

Encouragement. We express our sincere thanks to HVPGR for providing laboratory assistance and infrastructural facilities

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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DOI: 10.4172/2472-0992.1000112

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/61303

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(A journal of literary & educational)

ISSN : 2278-9189

Year : 7th, **Issue :** 34rd Janu - February-2020

Pages : 30, **Rs :** 50

Editor : Dr. D. M. Bhadresariya

Chief Editor : Harish Mangalam
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Publisher : DAMINEE PUBLICATIONS
A-22, Sonaparkshoping, Sonapark, I.O.C. Road, Chandkheda, Ahmedabad-382424. Gujarat. India. **Ph. : 91-9924721659**

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- પ્રા.બી.એસ.પટેલ

ગુજરાતી વિભાગ પી.એસ.સાયન્સ એન્ડ એચ.ડી.પટેલ આર્ટ્સ કૉલેજ, કડી.

અહીં ગુજરાતી રંગભૂમિના ઉદ્‌ભવ અંગે અભ્યાસ રજૂ કરવાનો ઉપક્રમ છે. રંગભૂમિ એ સમાજના જીવનઘડતરનું એક મહત્ત્વનું અંગ છે. લોકોના જીનને ઘડે છે અને નવો વળાંક આપે છે. લોકજીવનમાં એ અવનવા આદર્શો, ઉચ્ચ ભાવનાઓ અને સુંદર સંસ્કારો સિંચે છે. પ્રજાને એ પ્રેરે છે, સંસ્કારે છે અને ભવ્ય ભૂતકાળનું પ્રેરક દર્શન કરાવી ગૌરવ અર્પી ભવિ વિકાસનાં ઉજ્જવળ સોણલાં એ સિંચે છે. વળી વર્તમાન જીવનનું પ્રતિબિંબ દેખાડી સમાજની બદી અને કુરૂઢિઓને દફનાવવા માટે પણ લોકોને એ પ્રેરણા આપે છે. જાણીતા નાટકકાર ડાહ્યાભા ધોળશાજી ઝવેરીએ 'સતી પાર્વતી' નાટકના એક ગીતમાં યોગ્ય રીતે કહ્યું છે તેમ, ''નાટક તો ગુણદોષ જોવાનું, દિલડાનું દુઃખ ખોવાનું, ઘડીક હસાવતું, રડાવતું અને બોધ બતાવતું દુનિયાનું રૂડું દર્પણ છે.''

ગુજરાતી રંગભૂમિના ઉદ્‌ભવના અભ્યાસની અહીં બે તબક્કાઓમાં વિભાજિત કરી આપણે ચર્ચા કરીએ : ૧.ગુજરાતી રંગભૂમિનો પ્રથમ તબક્કો ૨. ગુજરાતી રંગભૂમિનો બીજો તબક્કો.

૧.ગુજરાતી રંગભૂમિનો પ્રથમ તબક્કો :

નાટકના પૂર્ણ સ્વરૂપ તરીકે ગુજરાતમાં પ્રચલિત વેશ ભવાંઈને ગણતરીમાં ન લઈએ તો પણ રસકવિ રઘુનાથ બ્રહ્મભટ્ટ યોગ્ય રીતે જણાવે છે તેમ વર્તમાન ગુજરાતી રંગભૂમિને આપણે જે સ્વરૂપમાં જોઈએ છીએ તેના ઉદ્‌ભવને લગભગ દોઢ સૈકા જેટલો સમય થઈ ગયો. કેટલાકનું મંતવ્ય એવું છે કે રંગભૂમિનો ઉદ્‌ભવ પ્રથમ મુંબઈને આંગણે થયો અને એના જન્મદાતા હતા મુંબઈના પારસીઓ. ત્યારે અંગ્રેજો 'બોમ્બે થિયેટર'માં જે શેક્સપિયર, મોલિયેર અને શેરિડનનાં નાટકો ભજવતાં હતાં તે જોઈને એવાં નાટકો ગુજરાતી અને ઉર્દૂ ભાષામાં ભજવવાની ઈચ્છાથી એમની જ શૈલીએ તેમણે નાટકો ભજવવાની શરૂઆત કરી અને પહેલું ગુજરાતી નાટક 'રૂસ્તમ સોહરાબ' ભજવ્યું. એમાં ગીતો કવિ દલપતરામે લખ્યાં હતાં. આમ, ઈ.સ.૧૮૪૩ના ઑક્ટોબરની પહેલી તારીખે પારસીઓએ 'પારસી નાટક મંડળી'ના નામથી ગુજરાતી રંગભૂમિનો પ્રારંભ કર્યો. આ શરૂઆત કરનાર હતા તે નાટક મંડળીના માલિક ફરામજી ગુ.દલાલ. આમ, ઈ.સ.૧૮૪૦-૧૮૫૦ની વચ્ચે ગુજરાતી નાટકો મુંબઈ, અમદાવાદ ને સુરતમાં ભજવાયાં હતાં.તે પછીથી ઈ.સ.૧૮૬૧ સુધીના અરસામાં જુદી જુદી પારસી નાટક મંડળીઓએ નાટકોની ધૂમ મચાવી. આવી નાટક મંડળીઓમાં મુખ્યત્વે પારસી નાટક મંડળી, એમેચ્યોર્સ ડ્રામેટિક કલબ, પારસી સ્ટેજ પ્લેયર્સ, જેન્ટલમેન નાટક મંડળી, શેક્સપિયર નાટક મંડળી, વાલિન્ટયર્સ કલબ, ઝોરાસ્ટિરયન ડ્રામેટિક સોસાયટી, એલ્ફિન્સ્ટન ડ્રામેટીક કલબ, વિક્ટોરિયા નાટક મંડળી, હિંદી નાટક મંડળી, પારસી વિક્ટોરિયા ઓપેરા મંડળી, આરિજીનલ વિક્ટોરિયા કલબ, ઓલ્ફિન્સ્ટન એમેચ્યોર્સ, રિપન નાટક મંડળી, આલ્ફ્રેડ નાટક મંડળી, પારસી એક્ષ્પ્રેસ વિક્ટોરિયા નાટક મંડળી અને મુંબઈ નાટક મંડળીઓનાં નામો ગણાવી શકાય. આ કંપનીઝએ 'ખૂને નાહક' ચતુરા

રણછોડરાય દવેકૃત 'લલિતા દુ:ખદર્શક', 'મદાલસા', 'જયકુમારી વિજય', 'ઋતુધ્વજ' અને 'બાણાસુર મદમર્દન' નાટકો ભજવ્યાં. આમ, ગુજરાતી રંગભૂમિના સ્થાપનાને પ્રેરણાને રણછોડરાય દવેનાં નાટકોથી વેગ મળ્યો અને તે ગુજરાતી રંગભૂમિના પ્રવર્તક તરીકે પંકાયા. તેમણે છ દશકા સુધી એકલે હાથે નાટ્યોપાસના કરી. એમનાં કુલ બાર નાટકો પ્રગટ થયાં છે. એ અગાઉ કવિ દલપતરામે ઈ.સ.૧૮૫૧માં 'લક્ષ્મી' નાટક ઈ.સ.૧૮૫૬માં 'સ્ત્રી સંભાષણ' નાટક અને ઈ.સ.૧૮૭૧માં 'મિથ્યાભિમાન' નાટક લખ્યાં હતાં. વળી, નવલરામ પંડ્યાએ ઈ.સ.૧૮૬૭માં 'ભટનું ભોપાળું' અને ઈ.સ.૧૮૬૯માં 'વીરમતી' નાટક લખ્યાં હતાં. પણ આ પૈકી એક પણ નાટક ત્યારે તખ્તા પર પહોંચ્યું નહોતું. પણ કવિ નર્મદે જે ઈ.સ.૧૮૬૯માં 'કૃષ્ણાકુમારી' ઈ.સ.૧૮૭૮માં 'દ્રૌપદી દર્શન', ઈ.સ.૧૮૭૬માં 'સીતા હરણ' અને 'સારશાકુંતલ' તથા ઈ.સ.૧૮૮૬માં 'બાલકૃષ્ણ વિજય' નામક નાટકો લખ્યાં હતાં તે પૈકી ઘણાં રંગભૂમિના તખ્તા પર ભજવયાં હતાં. આ રીતે નર્મદ આપણો પ્રથમ ધંધાદારી નાટકાર ગણાય. આ રીતે ગુજરાતી રંગભૂમિના વિકાસનો પહેલો તબક્કો પૂરો થયો.

ગુજરાતી રંગભૂમિના વિકાસના પહેલા તબક્કામાં પારસીઓએ રંગભૂમિનો આરંભ કર્યો અને હિંદુ ગુજરાતીઓએ એની જમાવટ કરી. આ અરસામાં ગુજરાતી નાટકોએ શાસ્ત્રીય સંગીતને લોકપ્રિય બનાવવામાં સંગીન ફાળો આપ્યો. સંગીત અને ગાયકો એ નાટકનાં મુખ્ય અંગ બન્યા. ઉર્દૂ નાટકો આદર્શ તરીકે અપનાવાયાં. બાહ્ય ઝળઝળાટને પ્રાધાન્ય મળ્યું. મનોરંજન તરફ ધ્યાન અપાયું. સ્થૂળ હાસ્યને નાટકના મૂળ કથાનક સાથે મહત્ત્વનું સ્થાન અપાયું. નાટ્યકાર ગૌણ ગણાયો અને નટ સર્વોપરી થયો. દલપતરામની કવિતાની અવગણના થઈ. સંચાલક નટ અને પ્રેક્ષકનું વર્ચસ્વ મહત્ત્વનું બનતાં નાટકકાર લાચાર સ્થિતિમાં મુકાઈ ગયો. હજુ સ્ત્રીઓએ રંગભૂમિ પર ભાગ લેવાનું શરૂ નહોતું કર્યું. એટલે સ્ત્રીપાત્રો બહુધા પુરુષો જ ભજવતા.

૨. ગુજરાતી રંગભૂમિનો બીજો તબક્કો :

ઈ.સ.૧૮૮૯માં 'મુંબઈ ગુજરાતી નાટક મંડળી'ની સ્થાપના શ્રી છોટાલાલ મૂળચંદ પટેલ, મગનલાલ મૂળચંદ પટેલ, દયાશંકર વ.પુરોહિત, કલ્યાણજી માવજી અને કિરપારામ નામના ચાર સજજનોએ કરી અને ગુજરાતી રંગભૂમિના વિકાસનો બીજો તબક્કો શરૂ થયો. સાક્ષર શ્રી મણિલાલ ન.દ્વિવેદીના 'કાન્તા' નાટકને 'કુલીન કાન્તા' નામ આપી તથા ભજવી એ મંડળીએ પોતાની નાટ્યપ્રવૃત્તિ શરૂ કરી. મૂળશંકર મૂલાણીકૃત 'મૂળરાજ સોલંકી' નાટકથી એને ઘણી લોકપ્રિયતા મળી. એનાં મધુર ગીતો અત્યારે અંત્યત પ્રિય બન્યાં હતાં. એ ઉપરાંત ચંદા, સુંદરવેણી, કનકતારા, સૌભાગ્યનો સિંહ, દેવકન્યા, શકુન્તલા, મેઘમાલિની, કરણઘેલો, રાજબીજ. જુગલ જુમારી, વિક્રમ ચરિત્ર, સૌભાગ્ય સુંદરી, અજબ કુમારી, પૃથ્વીરાજ ચૌહાણ, નંદબત્રીસી, વસંતપ્રભા, મધુબંસરી, સુધાચંદ્ર, હરિશ્ચંદ્ર અને સ્નેહસરિતા આદિ એનાં નાટકો લોકોમાં પ્રિય થઈ પડ્યાં હતાં. એમાંય 'કરણોઘેલો', 'વિક્રમચરિત્ર' અને 'સૌભાગ્ય સુંદરી' નાટકોએ મંડળીને ખૂબ જ પ્રતિષ્ઠા અપાવી હતી. મંડળીએ બીજા નાટકોમાં ''મંદાધમહિલા, કુમળી કળી, જમાનાનો રંગ, તરૂણીના તરંગ, આજની દુનિયા, નારી હૃદય, લક્ષ્મીના લોભે, રાજાધિરાજ, કાશ્મીરનું પ્રભાત, કુદરતનો ન્યાય,

Preparation and Characterization of Penta Allyl Sucrose

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Abstract

Allyl sucrose was prepared by condensation reaction among sucrose, allyl chloride, NaOH and DMSO which makes suspension. The employed method is new, faster, consumes less solvent and more convenient to isolate product than the existing methods. There is no need of column chromatography. The yield of synthesized product is 90%.

The prepared product is characterized by various spectral studies such as FT-IR (Fourier-Transform Infrared), NMR (Nuclear Magnetic Resonance) and Mass spectrometry. It is used in different industrial fields.

Keywords: Allyl chloride, allyl sucrose, cross-linking agent, FT-IR, NMR, Mass.

Introduction

Allyl sucrose can be in use as a cross linking agent in synthesis of co-polymers. The various cross linking agents are in work for the copolymerization of poly acrylic acids to optimize the thickening properties of the polymers. Allyl sucrose has become an important thickening agent for the preparation of the variety of polymer applied in many industrial fields, such as coating material, medicines, cosmetics, epoxy resins etc.[1-4] These compounds can be used as film- forming materials/plastic compositions which are on polymerization yield products that are highly resistant to the action of heat, solvents and other reagents. It is also used as an up grader of drying and semidrying oils[5-13]. Various carbohydrates are used in preparation of cross-linkers like starch, cellulose etc. described in various patents[14-18].

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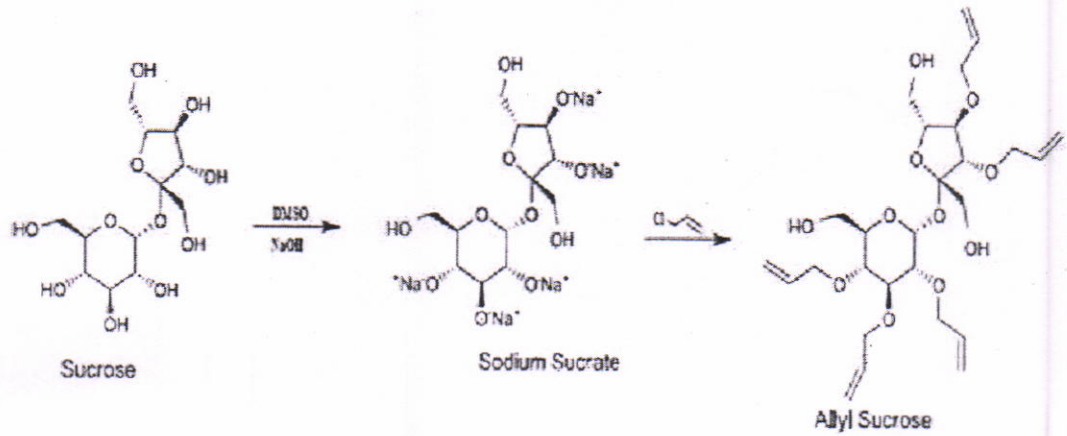

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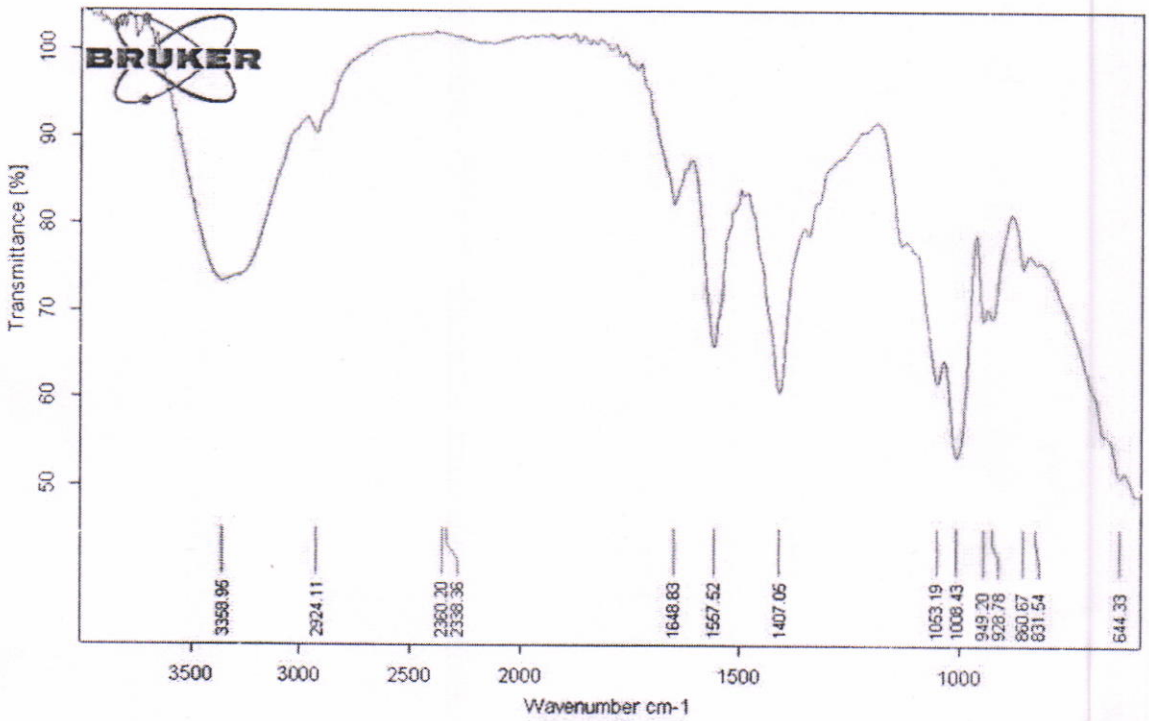

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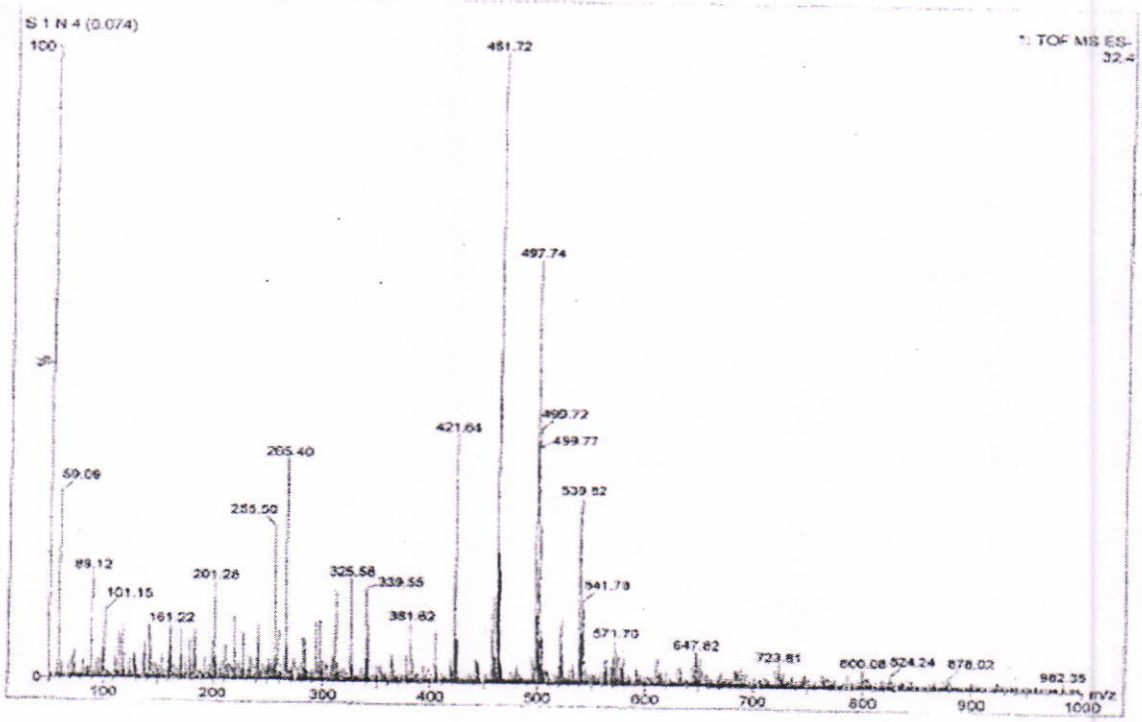

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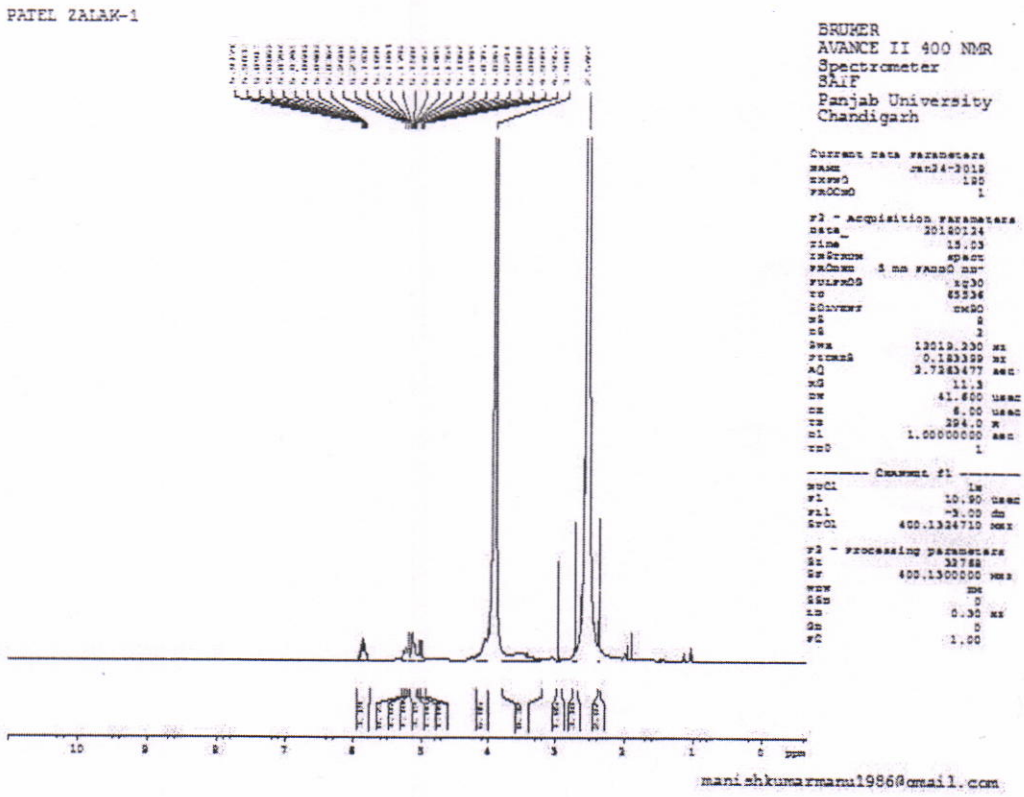

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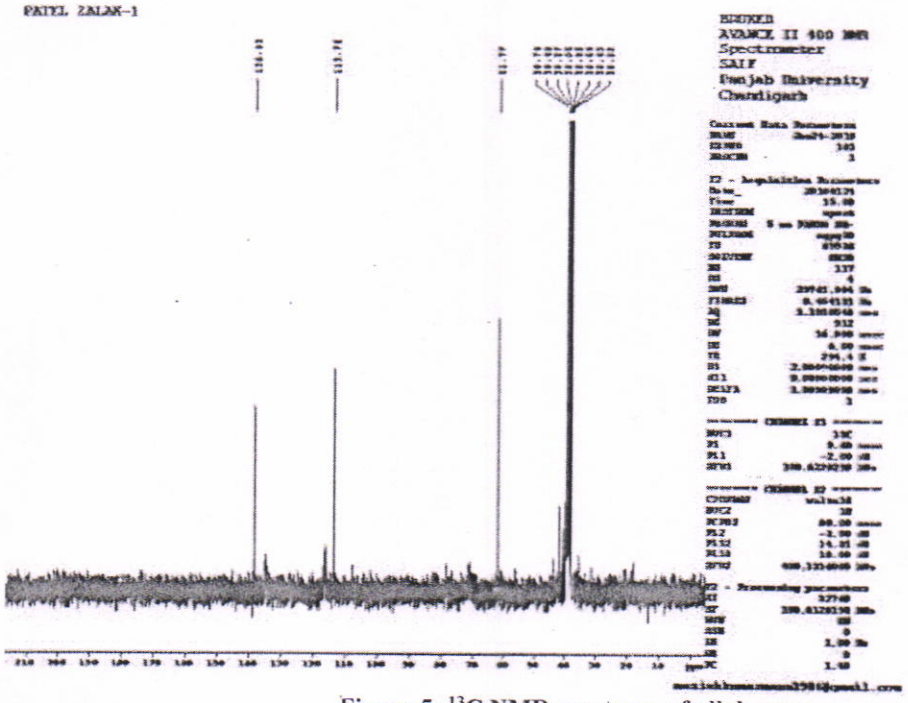

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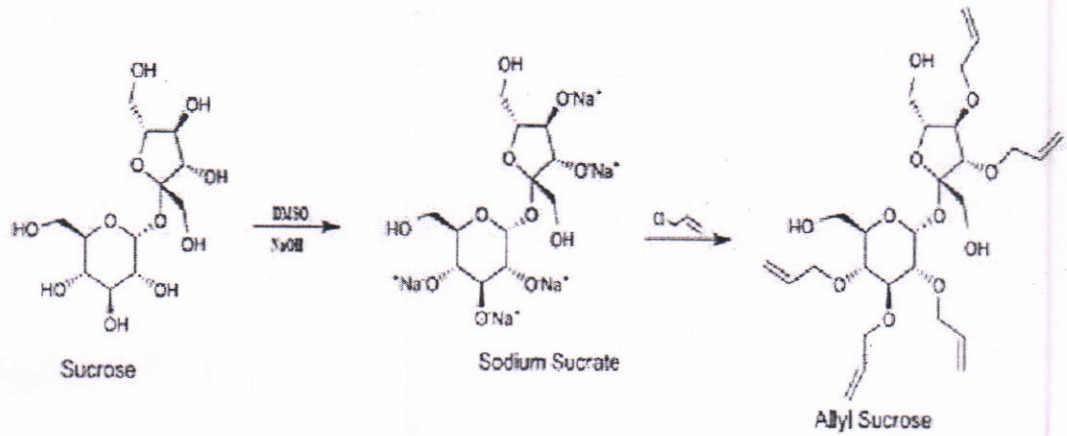

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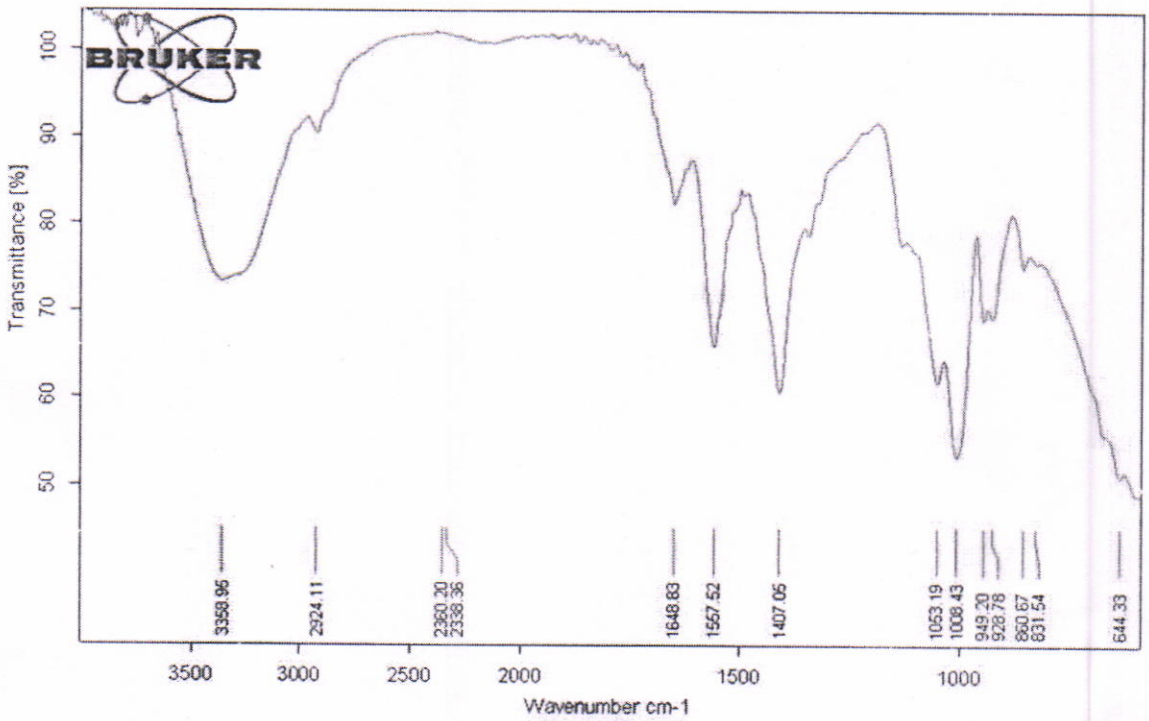

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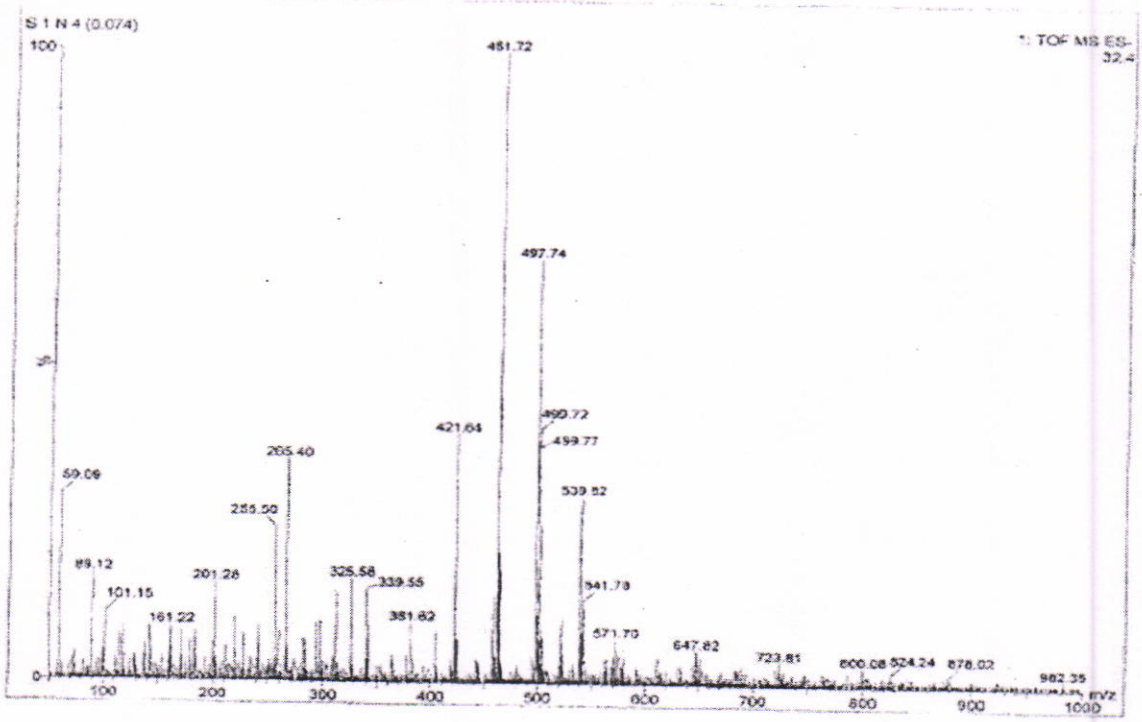

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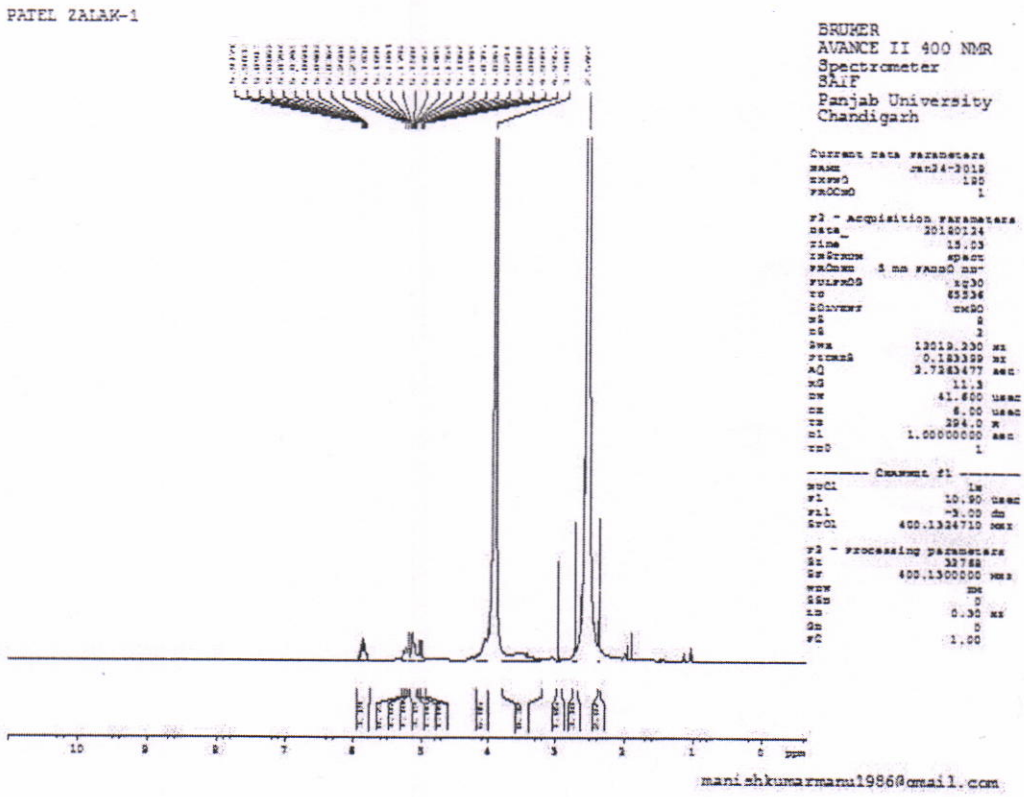

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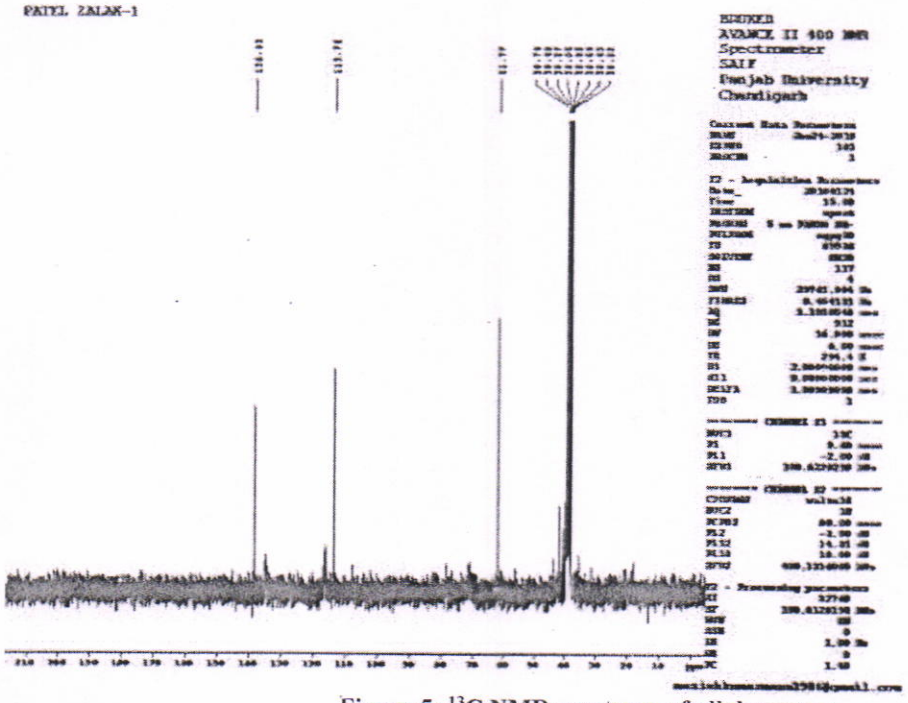

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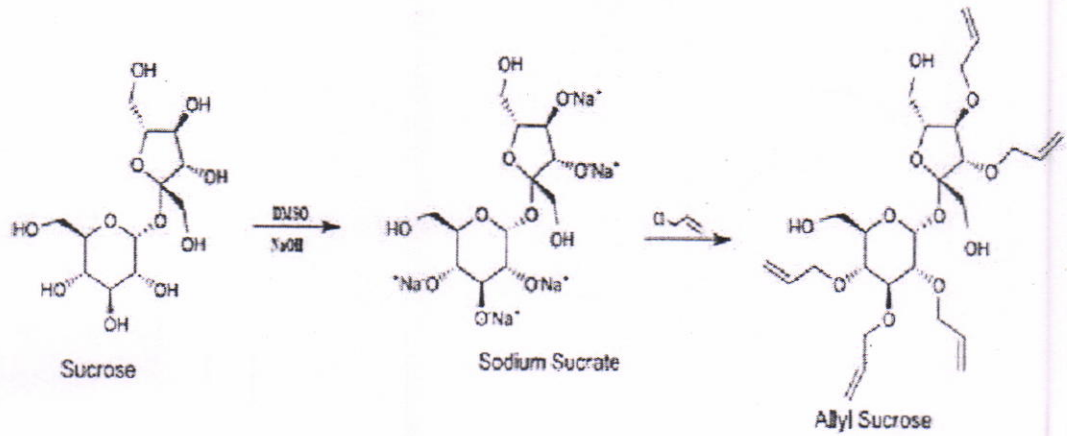

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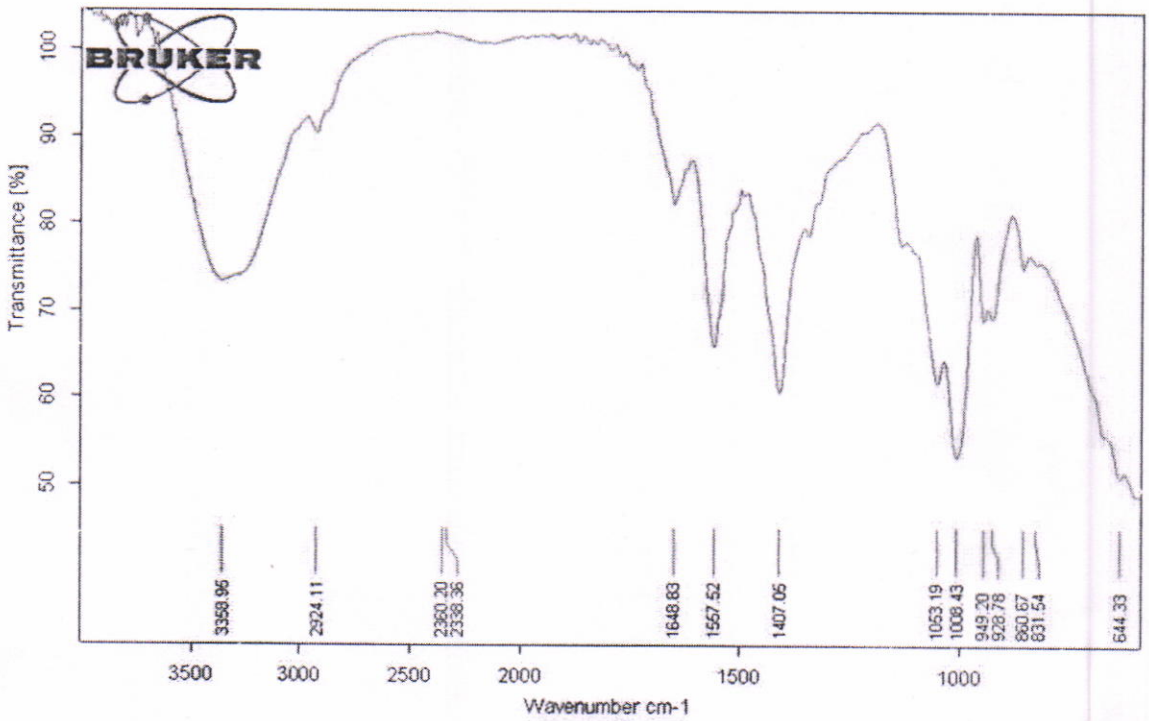

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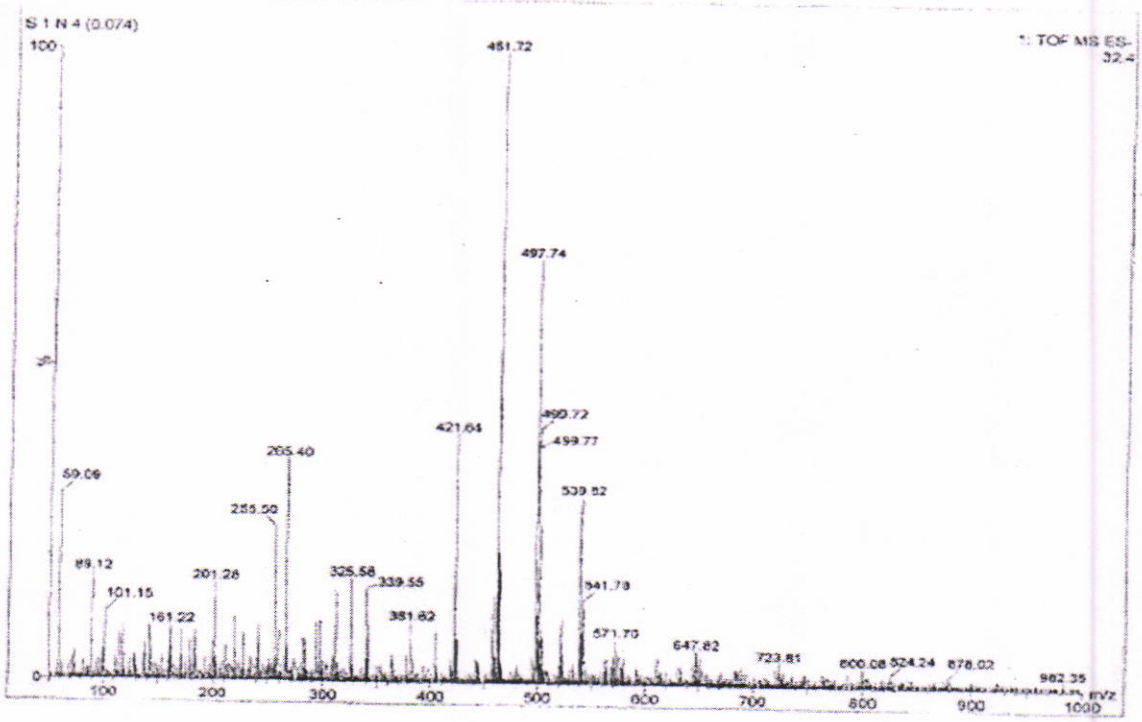

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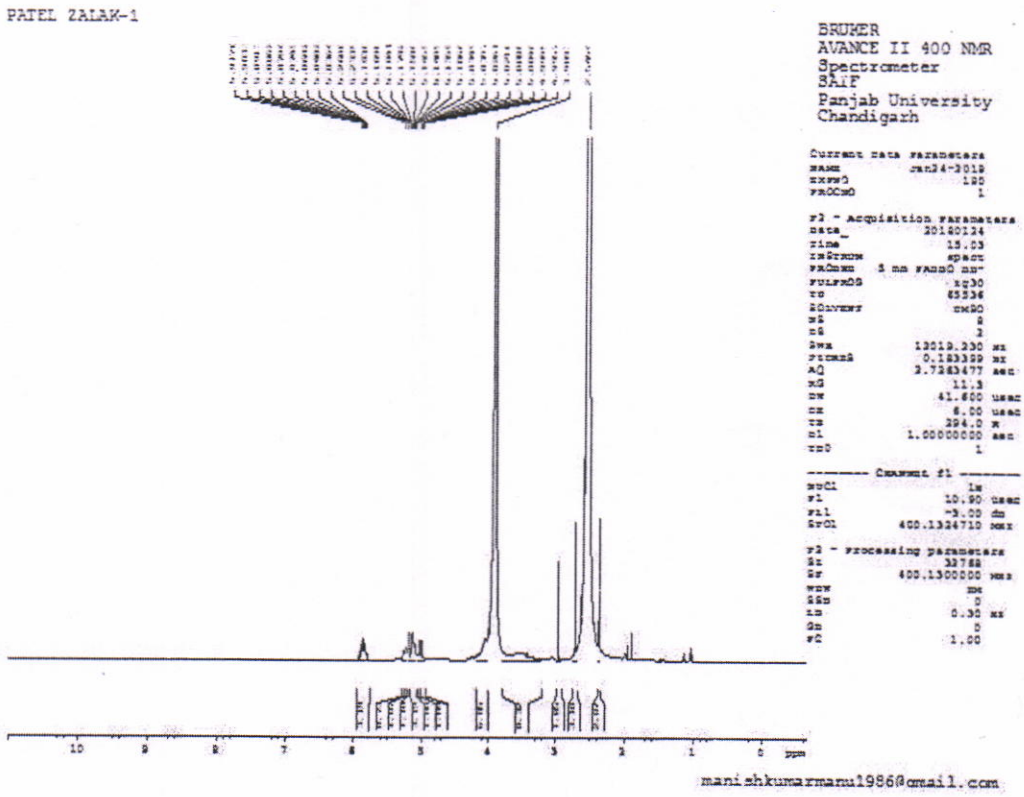

Figure 4: ^{1}H NMR spectrum of allyl sucrose

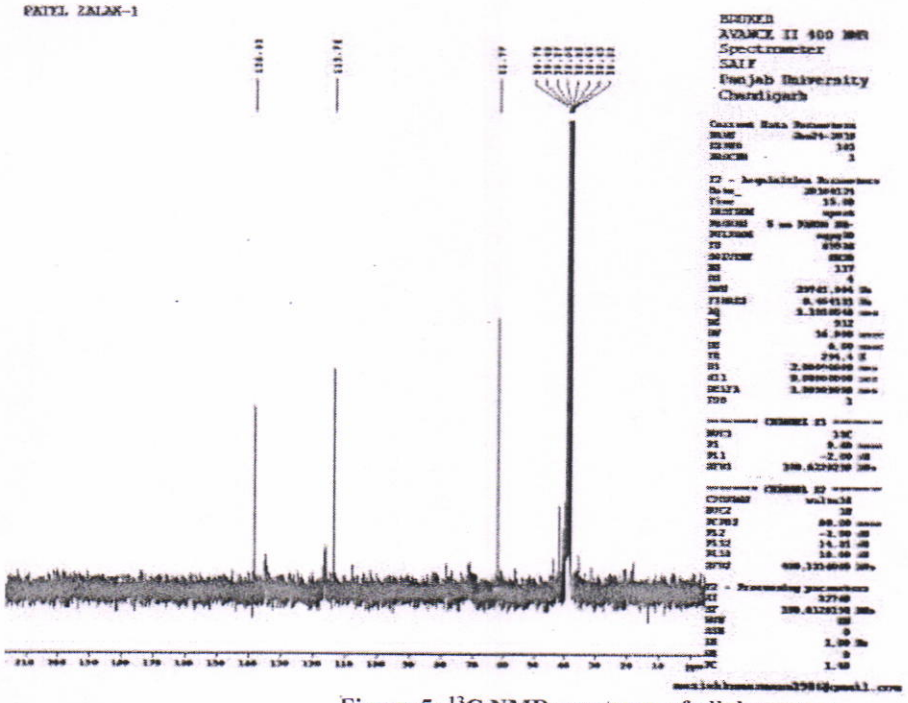

Figure 5: ^{13}C NMR spectrum of allyl sucrose

Table 1
Spectral data of allyl sucrose

S.N.	Absorption Range [cm^{-1}]	Type of Vibration
1	3358	O-H Stretching
2	2924	C = C-H Stretching
3	1648, 1557	C = C Stretching of allyl group
4	1053	C-O Stretching
5	1008	C = C-H Bending of allyl
6	928	C = C-H Bending of allyl

Conclusion

From the resultant data of mass spectra it is clear that five allyl groups are attached to a sucrose molecule. This method is more rapid, economically efficient and has no difficulty of isolation than the available methods. The present method is also used on large scale industrial preparation.

Acknowledgement

We acknowledge thanks to Research Laboratory, Pramukh Swami Science and H D Patel Arts College, Kadi for providing research facilities.

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(Received 28th August 2019, accepted 22nd October 2019)

Synthesis and characterization of novel swollen cross-linked poly acrylic acid

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Abstract

A cross-linked poly acrylic acid super absorbent polymer has been successfully synthesized by reacting poly allyl sucrose, styrene and acrylic acid batch charging in aqueous medium under the occurrence of nitrogen atmosphere. This novel method is profitable in terms of reducing the toxicity and is fast and economical than available methods. The morphological structure of the synthesized polymer was studied by SEM which showed layered self-assembled structures.

Using FTIR, the characteristically involved functional groups of the functionalized polymers were identified. To analyze the thermal character of the prepared polymer in terms of their endothermic or exothermic behavior, TGA and DSC techniques were employed. The synthesized materials could be used as water-absorbing agents in the field of material science and technology.

Keywords: Poly allyl sucrose, styrene, acrylic acid, water-absorbing agent, particle size, copolymer composition, TGA, DSC, FT-IR, SEM.

Introduction

Superabsorbent polymers (SAPs) are cross-linked polymers which absorb and retain a great amount of water compared to its own mass. There are numerous types of SAP including poly (acrylic acid) (PAA), poly acrylamide (PAAm), starch-graft-poly acrylonitrile (PAN)[1] and cross-linked carboxymethyl cellulose[2]. Universal SAP manufacture capability reached 3.5 million tons in year 2015[3]. At present, the major market of SAP is baby diapers and other hygiene products such as adult incontinence products, hygienic napkins and absorption pads for hospital uses. Additional applications of SAPs comprise soil moisture conditioning, aqueous waste treatment, cable water jamming and tangible additives etc.[4]

Excessively retentive polymers are cross-connected polymer contain network like a chain without dissolving and they can retain just as grip the enormous amount of water in the swollen structure. The retained fluid is barely removable still under certain powers[5]. Super sponges have perceived critical thought as a result of their impressive applications in few zones, for example, sterile products[6-8], gardening[9], squander water treatment[10] and prescription for medication-free strategy[11]while in a wide assortment of uses, about all of the super sponges utilized as dispensable particles depend on completely oil-based polymers with high creation cost and having genuine condition sway and along these lines, they have certain limits. In water, SAPs swell to a straightforward chewy gel and their mass can suck up and hold unforeseen extraordinary measures of water or fluid arrangement[12].

The making of the primary water-spongy polymer organized from acrylic acid (AA) and the cross-connecting agent styrene was thermally polymerized in a fluid medium and afterward the main development of hydrogels was shown up[13]. These hydro gels were generally found on hydroxy alkyl methacrylate and associated monomers with swelling ability up to 50%. In the present strategy, water is utilized as a dissolvable medium and $K_2S_2O_8$ as an initiator. This strategy is useful in completing the enormous number of polymer items in lesser time when contrasted with the ordinary strategies. Water is picked as dissolvable in this polymerization response to make blend greener way.

Material and Methods

Chemicals and equipments: Allyl sucrose, styrene, acrylic acid, ethanol and $K_2S_2O_8$ are of laboratory grade. Basic glass products like three-neck round bottom flask and gear like mechanical stirrer and Brooker FT-IR spectrophotometer were used.

Synthesis of salts of cross-linked poly acrylic acid: A nitrogen gas gulf tube and mechanical stirrer were fitted with a three necked round bottom flask of 1000 ml limit. 100 ml of dissolvable blend is moved into the flask and includes two little bits of porcelain. Gradually heat the dissolvable blend to 60 °C temperature and afterward pass the nitrogen gas to sparge the flask substance to power out the air. Allyl sucrose (1, 0.8, 0.6, 0.4, 0.2 g), a diverse measure of acrylic acid and styrene are added to the response blend and mixed. In the meantime, 1 % of an initiator ($K_2S_2O_8$) is added to start the polymerization and temperature is step by step expanded to 80°C. Jellified item is seen in 45 minutes. The gelisdried for 24 hours and then granulated for further assessment. The reaction mixture is employed is as described in table 1.

Swelling test: SAP (0.5 g) samples (W_0) are placed into a pre-weighed beaker. The polymers are dipped in an excess amount of water for overnight to reach the stability of swelling. Then the excess amount of water is removed until

no liquid was dropped off. The polymers are weighed (W_1) and the swelling capacity was calculated by equation:

Swelling capacity = Wet/dry*100% = W_1/W_0*100%

Absorbance capacity of polymers is shown in table 2.

Results and Discussion

FT-IR spectra: The Fourier Transform Infrared (Brooker FT–IR spectrometer alpha Model) Spectroscopic technique is used for characterization of polymers by preparing KBr pellets of the materials. The representative IR spectrum of the polymer is displayed in figure 1. The spectra exhibit the characteristic IR peak at 3010 cm^{-1}(-C=C-H stretching) of CH_2 scissoring and the strong peak at 1696 cm^{-1} for –C=O stretching of inter-molecular hydrogen bonding of acrylic acid. The absorption band at 1188 cm^{-1} is recognized for –C - O stretching of ether bending vibration. The peak at 1395 cm^{-1} is accredited to O-H bending vibration.

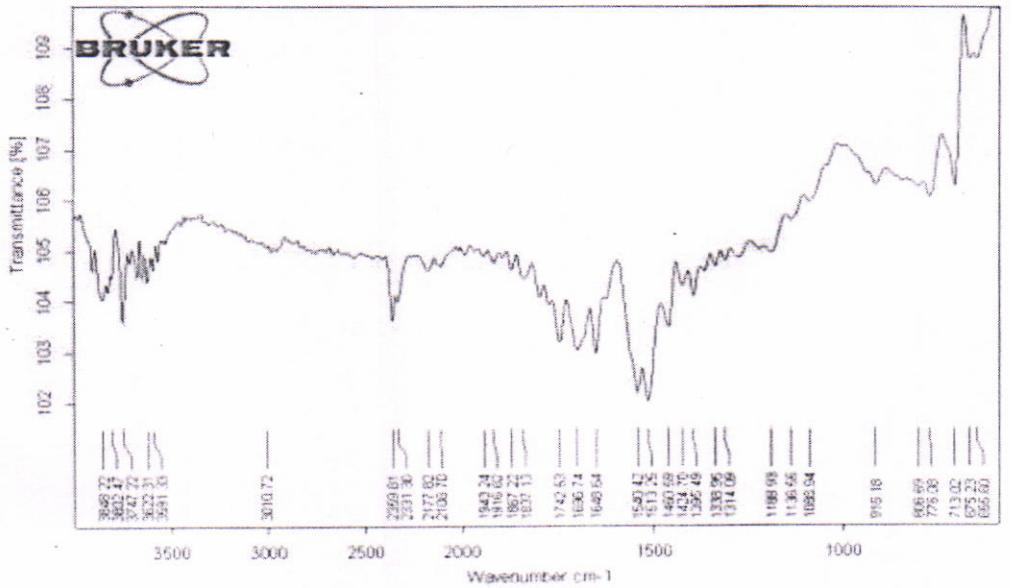

Fig. 1: FT-IR of cross-linked polymer

Table 1
Amount of reagents

CHEMICALS	AMOUNT (2.1)	AMOUNT (2.2)	AMOUNT (2.3)	AMOUNT (2.4)	AMOUNT (2.5)
Allyl sucrose	1 gm	0.8 gm	0.6 gm	0.4 gm	0.2 gm
Styrene	56 gm	56.1 gm	56.2 gm	56.3 gm	56.4 gm
Acrylic acid	43 gm	43.1 gm	43.2 gm	43.3 gm	43.4 gm
Ini.– $K_2S_2O_8$	1 gm	1 gm	1 gm	1 gm	1 gm
Sol.-H_2O+C_2H_5OH	490 + 10 ml	490 + 10 ml	490 + 10 ml	490 + 10 ml	490 + 10 ml
Neut. - NaOH	7 pH	7 pH	7 pH	7 pH	7 pH

Table 2
Absorbance capacity of polymer

Wt. of cross-linking agent	Weight of sample (g)	Weight of swelled polymer (g)	Swelling capacity %
1.0 g	0.500 g	11.760	2252.13
0.8 g	0.500 g	26.119	5123.81
0.6 g	0.500 g	1.4280	185.612
0.4 g	0.500 g	44.255	8750.97
0.2 g	0.500 g	20.903	4080.69

Thermal analysis: The thermo gravimetric analysis (TGA) of the polymers indicates (Figure 2-6) that degradation of a polymer starts around 150°C and degrades completely above 200°C to 260°C. So, the polymers are thermally stable up to 150°C. The canyons (converse peaks) are due the fact that polymer has started to degrade and heat is liberated during degradation.

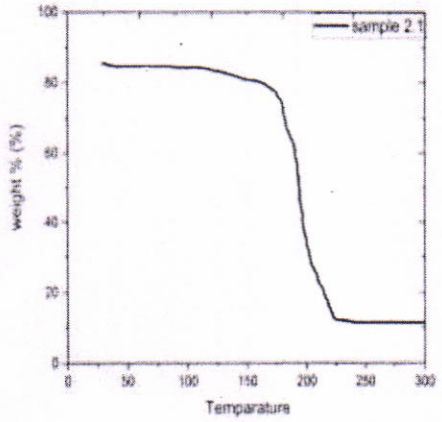

Fig. 2: TGA of cross-linked polymer (2.1)

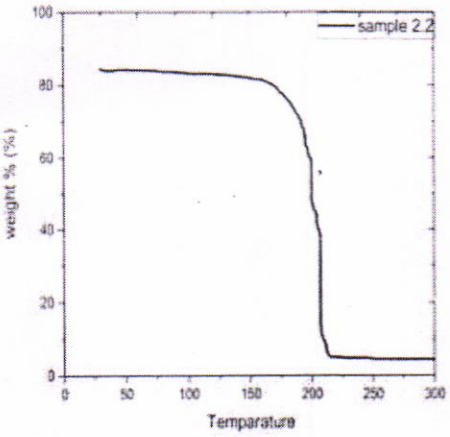

Fig. 3: TGA of cross-linked polymer (2.2)

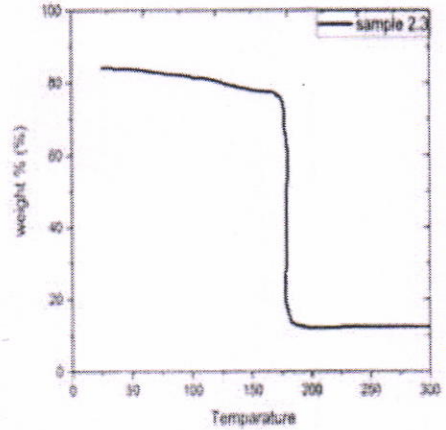

Fig. 4: TGA of cross-linked polymer (2.3)

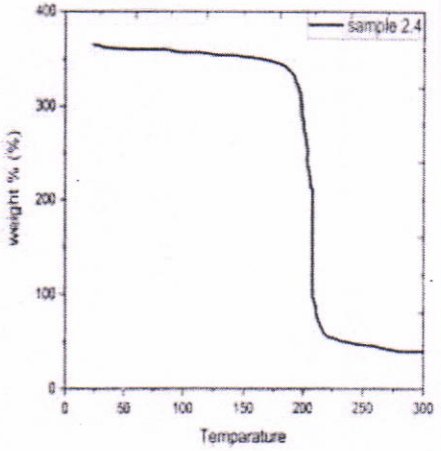

Fig. 5: TGA of cross-linked polymer (2.4)

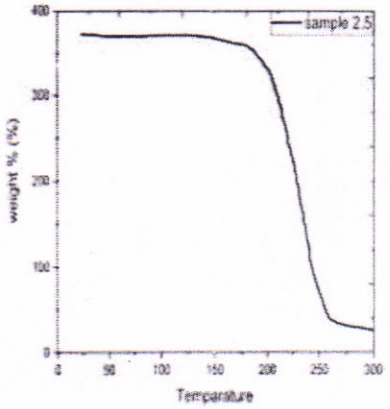

Fig. 6: TGA of cross-linked polymer (2.5)

Differential Scanning Calorimetric (DSC) analysis: The cross-linking is also characterized by DSC analysis as shown in figure 7-11. DSC of SAP cross-linked shows a weight loss within two stages. Essentially, acrylic acid has the glass-transition temperature *(T_g)* about 106°C, but due to cross-linked with styrene, there may be slightly change in T_g. DSC thermograms designate that T_g of the polymer increases by about 115°C and is independent of the amount of allyl sucrose. There is the hump in the bend and fix the midpoint in each curve as T_g. The initial stage shows weight loss between 40°C and 125°C which may be due to the loss of absorbed and bound water. The next stage of mass loss started at 225°C and continued to 275°C during which weight loss may due to the degradation of the cross-linker.

Morphological Analysis: To investigate surface morphology of the synthesized polymer, SEM analysis was carried out for the sample. Figure 12a shows the clear surface morphology of the synthesized sample at different scale. Figure 12 scanned at 200 µm shows a fiber like amorphous structures including semi solid particles. The higher resolution of the analyzed image indicates multilayer

polymeric structures having random particles which may be present due to the presence of acrylic acids and sucrose molecules (figure 12 b). However, figure 12c shows randomly distributed polymeric nonporous structures which are finely scattered with same morphology.

Conclusion

It is well observed that the allyl sucrose and acrylic acid are hydrophilic, but styrene is hydrophobic which is fundamentally used in our reported work. Polymer segments of acrylic acid/styrene form one phase and ally sucrose with acrylic acid form another immiscible phase. In this work, by using this method clear rubbery gel is achieved. DSC of SAP cross-linked with AS shows a weight loss.

The predictable super absorbent polymer synthesized from acrylic acid and cross-linking agent has a solid smooth non-porous surface. The cross-linking was also supported by DSC analysis.

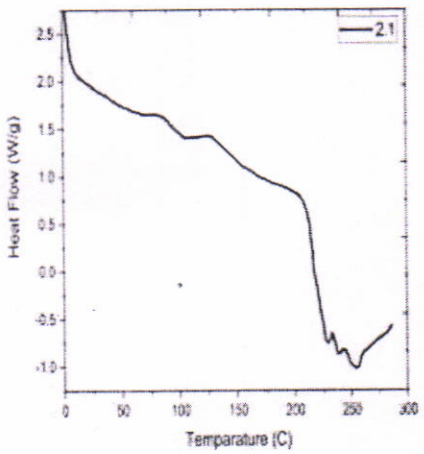

Fig. 7: DSC of cross-linked polymer (2.1)

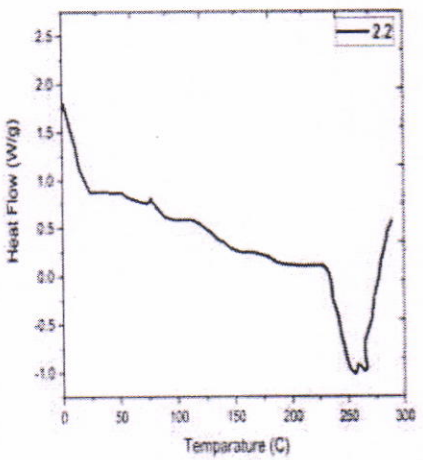

Fig. 8: DSC of cross-linked polymer (2.2)

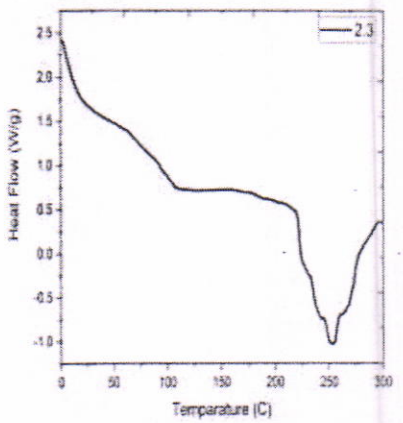

Fig. 9: DSC of cross-linked polymer (2.3)

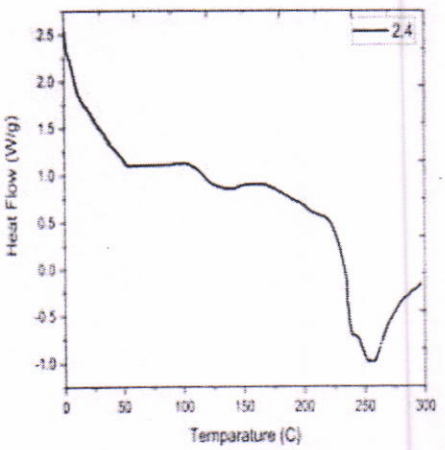

Fig. 10: DSC of cross-linked polymer (2.4)

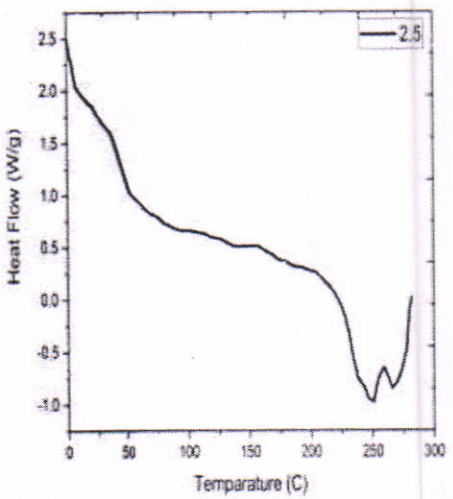

Fig. 11: DSC of cross-linked polymer (2.5)

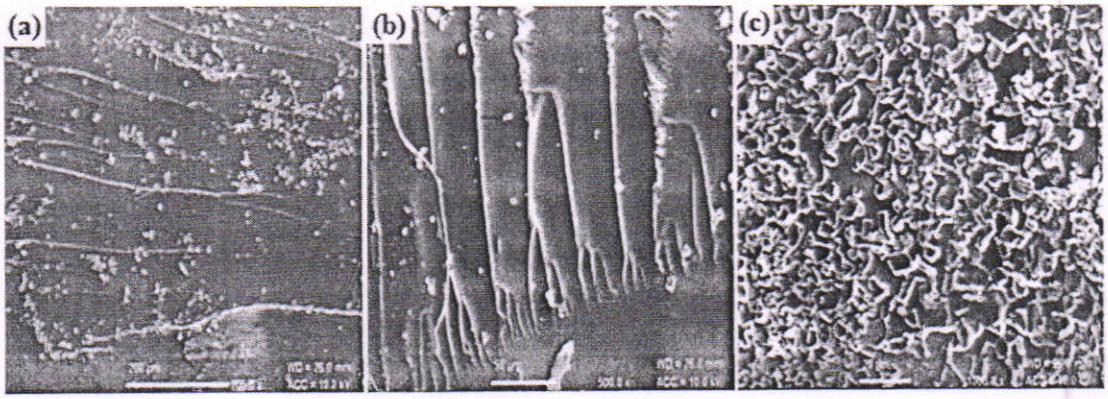

Fig. 12: SEM micrographs of prepared SAP's show superabsorbent polymer cross-linked with PAA-AS and styrene with different micro sizes

Hydrophilic networks reactive to some molecules can be used in drug systems and in controlled release drugs[10]. Super absorbent polymers were also employed in various applications such as absorbent paper products, bandages and surgical pads, wound dressings and as chemical absorbents. Additionally, they are applicable in food packaging[15]. From the swelling analysis, it is investigated that the newly invented polymers absorbed higher amount of water content.

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(Received 03rd September 2019, accepted 05th November 2019)

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Abstract

A cross-linked poly acrylic acid super absorbent polymer has been successfully synthesized by reacting poly allyl sucrose, styrene and acrylic acid batch charging in aqueous medium under the occurrence of nitrogen atmosphere. This novel method is profitable in terms of reducing the toxicity and is fast and economical than available methods. The morphological structure of the synthesized polymer was studied by SEM which showed layered self-assembled structures.

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Keywords: Poly allyl sucrose, styrene, acrylic acid, water-absorbing agent, particle size, copolymer composition, TGA, DSC, FT-IR, SEM.

Introduction

Superabsorbent polymers (SAPs) are cross-linked polymers which absorb and retain a great amount of water compared to its own mass. There are numerous types of SAP including poly (acrylic acid) (PAA), poly acrylamide (PAAm), starch-graft-poly acrylonitrile (PAN)[1] and cross-linked carboxymethyl cellulose[2]. Universal SAP manufacture capability reached 3.5 million tons in year 2015[3]. At present, the major market of SAP is baby diapers and other hygiene products such as adult incontinence products, hygienic napkins and absorption pads for hospital uses. Additional applications of SAPs comprise soil moisture conditioning, aqueous waste treatment, cable water jamming and tangible additives etc.[4]

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no liquid was dropped off. The polymers are weighed (W_1) and the swelling capacity was calculated by equation:

Swelling capacity = Wet/dry*100% = W_1/W_0*100%

Absorbance capacity of polymers is shown in table 2.

Results and Discussion

FT-IR spectra: The Fourier Transform Infrared (Brooker FT–IR spectrometer alpha Model) Spectroscopic technique is used for characterization of polymers by preparing KBr pellets of the materials. The representative IR spectrum of the polymer is displayed in figure 1. The spectra exhibit the characteristic IR peak at 3010 cm^{-1}(-C=C-H stretching) of CH_2 scissoring and the strong peak at 1696 cm^{-1} for –C=O stretching of inter-molecular hydrogen bonding of acrylic acid. The absorption band at 1188 cm^{-1} is recognized for –C - O stretching of ether bending vibration. The peak at 1395 cm^{-1} is accredited to O-H bending vibration.

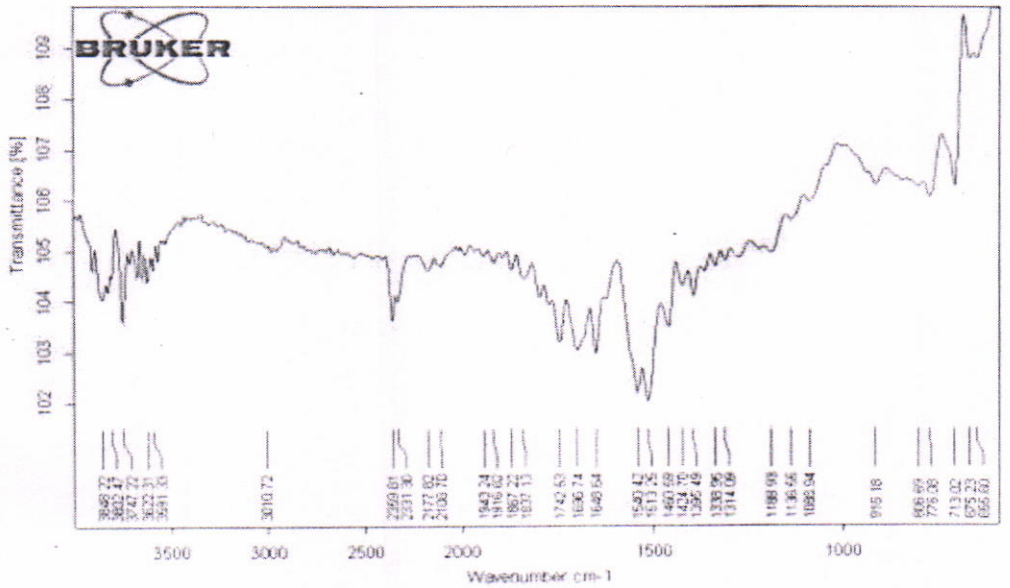

Fig. 1: FT-IR of cross-linked polymer

Table 1
Amount of reagents

CHEMICALS	AMOUNT (2.1)	AMOUNT (2.2)	AMOUNT (2.3)	AMOUNT (2.4)	AMOUNT (2.5)
Allyl sucrose	1 gm	0.8 gm	0.6 gm	0.4 gm	0.2 gm
Styrene	56 gm	56.1 gm	56.2 gm	56.3 gm	56.4 gm
Acrylic acid	43 gm	43.1 gm	43.2 gm	43.3 gm	43.4 gm
Ini.– $K_2S_2O_8$	1 gm	1 gm	1 gm	1 gm	1 gm
Sol.-H_2O+C_2H_5OH	490 + 10 ml	490 + 10 ml	490 + 10 ml	490 + 10 ml	490 + 10 ml
Neut. - NaOH	7 pH	7 pH	7 pH	7 pH	7 pH

Table 2
Absorbance capacity of polymer

Wt. of cross-linking agent	Weight of sample (g)	Weight of swelled polymer (g)	Swelling capacity %
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0.2 g	0.500 g	20.903	4080.69

Thermal analysis: The thermo gravimetric analysis (TGA) of the polymers indicates (Figure 2-6) that degradation of a polymer starts around 150°C and degrades completely above 200°C to 260°C. So, the polymers are thermally stable up to 150°C. The canyons (converse peaks) are due the fact that polymer has started to degrade and heat is liberated during degradation.

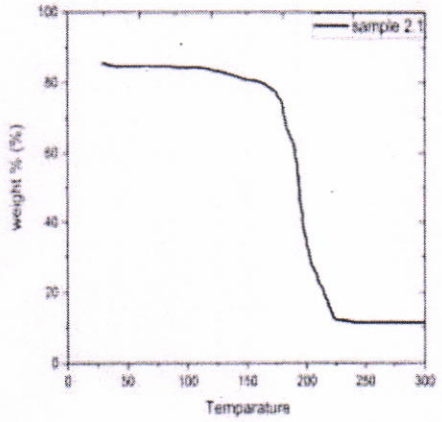

Fig. 2: TGA of cross-linked polymer (2.1)

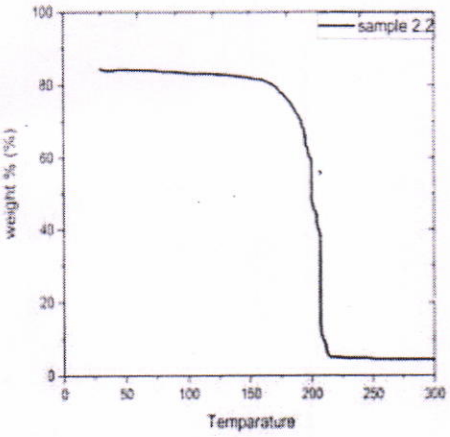

Fig. 3: TGA of cross-linked polymer (2.2)

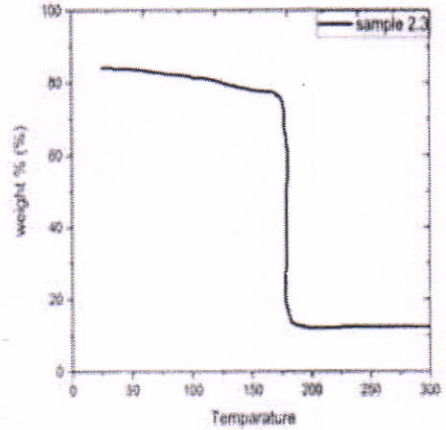

Fig. 4: TGA of cross-linked polymer (2.3)

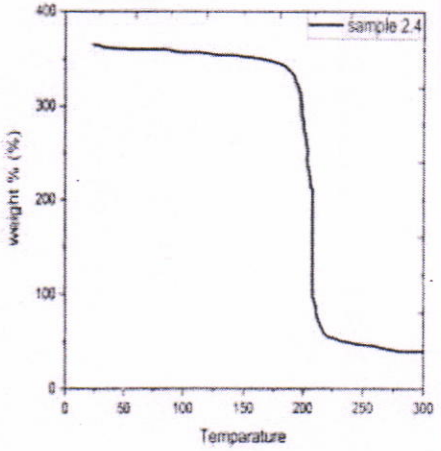

Fig. 5: TGA of cross-linked polymer (2.4)

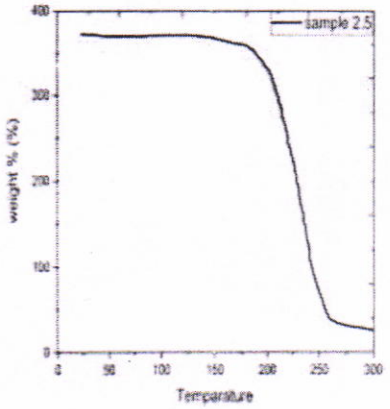

Fig. 6: TGA of cross-linked polymer (2.5)

Differential Scanning Calorimetric (DSC) analysis: The cross-linking is also characterized by DSC analysis as shown in figure 7-11. DSC of SAP cross-linked shows a weight loss within two stages. Essentially, acrylic acid has the glass-transition temperature *(T_g)* about 106°C, but due to cross-linked with styrene, there may be slightly change in T_g. DSC thermograms designate that T_g of the polymer increases by about 115°C and is independent of the amount of allyl sucrose. There is the hump in the bend and fix the midpoint in each curve as T_g. The initial stage shows weight loss between 40°C and 125°C which may be due to the loss of absorbed and bound water. The next stage of mass loss started at 225°C and continued to 275°C during which weight loss may due to the degradation of the cross-linker.

Morphological Analysis: To investigate surface morphology of the synthesized polymer, SEM analysis was carried out for the sample. Figure 12a shows the clear surface morphology of the synthesized sample at different scale. Figure 12 scanned at 200 µm shows a fiber like amorphous structures including semi solid particles. The higher resolution of the analyzed image indicates multilayer

polymeric structures having random particles which may be present due to the presence of acrylic acids and sucrose molecules (figure 12 b). However, figure 12c shows randomly distributed polymeric nonporous structures which are finely scattered with same morphology.

Conclusion

It is well observed that the allyl sucrose and acrylic acid are hydrophilic, but styrene is hydrophobic which is fundamentally used in our reported work. Polymer segments of acrylic acid/styrene form one phase and ally sucrose with acrylic acid form another immiscible phase. In this work, by using this method clear rubbery gel is achieved. DSC of SAP cross-linked with AS shows a weight loss.

The predictable super absorbent polymer synthesized from acrylic acid and cross-linking agent has a solid smooth non-porous surface. The cross-linking was also supported by DSC analysis.

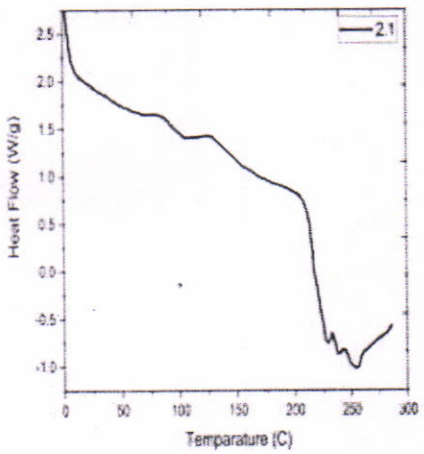

Fig. 7: DSC of cross-linked polymer (2.1)

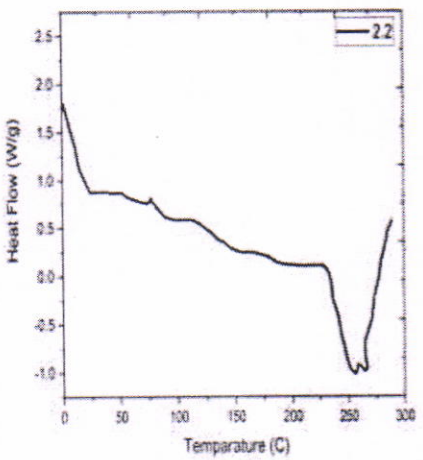

Fig. 8: DSC of cross-linked polymer (2.2)

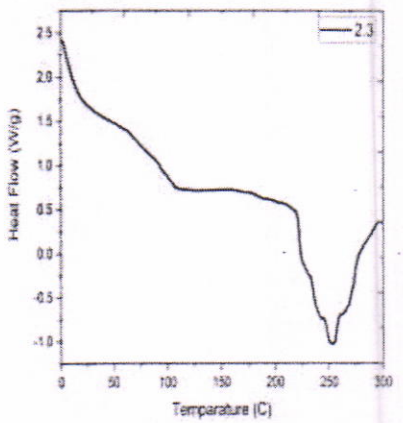

Fig. 9: DSC of cross-linked polymer (2.3)

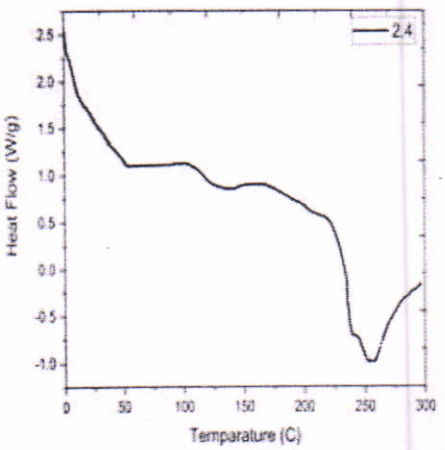

Fig. 10: DSC of cross-linked polymer (2.4)

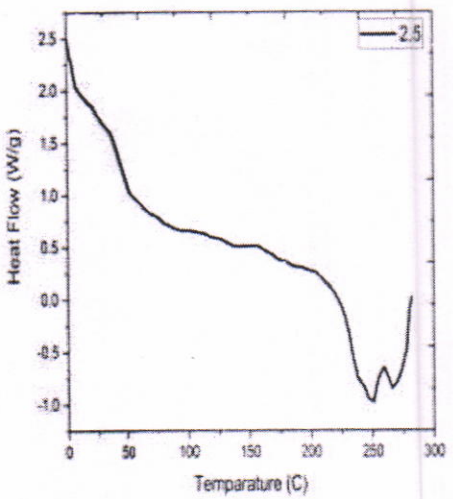

Fig. 11: DSC of cross-linked polymer (2.5)

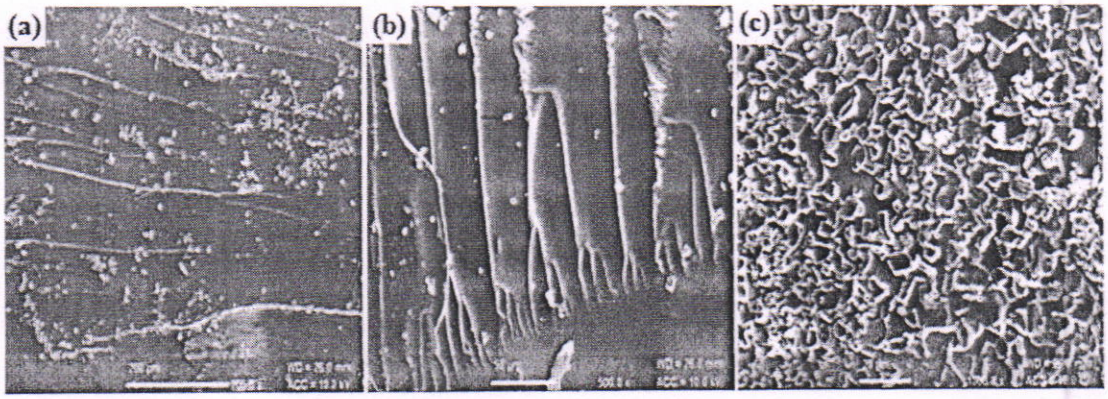

Fig. 12: SEM micrographs of prepared SAP's show superabsorbent polymer cross-linked with PAA-AS and styrene with different micro sizes

Hydrophilic networks reactive to some molecules can be used in drug systems and in controlled release drugs[10]. Super absorbent polymers were also employed in various applications such as absorbent paper products, bandages and surgical pads, wound dressings and as chemical absorbents. Additionally, they are applicable in food packaging[15]. From the swelling analysis, it is investigated that the newly invented polymers absorbed higher amount of water content.

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(Received 03rd September 2019, accepted 05th November 2019)

Synthesis and characterization of novel swollen cross-linked poly acrylic acid

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Abstract

A cross-linked poly acrylic acid super absorbent polymer has been successfully synthesized by reacting poly allyl sucrose, styrene and acrylic acid batch charging in aqueous medium under the occurrence of nitrogen atmosphere. This novel method is profitable in terms of reducing the toxicity and is fast and economical than available methods. The morphological structure of the synthesized polymer was studied by SEM which showed layered self-assembled structures.

Using FTIR, the characteristically involved functional groups of the functionalized polymers were identified. To analyze the thermal character of the prepared polymer in terms of their endothermic or exothermic behavior, TGA and DSC techniques were employed. The synthesized materials could be used as water-absorbing agents in the field of material science and technology.

Keywords: Poly allyl sucrose, styrene, acrylic acid, water-absorbing agent, particle size, copolymer composition, TGA, DSC, FT-IR, SEM.

Introduction

Superabsorbent polymers (SAPs) are cross-linked polymers which absorb and retain a great amount of water compared to its own mass. There are numerous types of SAP including poly (acrylic acid) (PAA), poly acrylamide (PAAm), starch-graft-poly acrylonitrile (PAN)[1] and cross-linked carboxymethyl cellulose[2]. Universal SAP manufacture capability reached 3.5 million tons in year 2015[3]. At present, the major market of SAP is baby diapers and other hygiene products such as adult incontinence products, hygienic napkins and absorption pads for hospital uses. Additional applications of SAPs comprise soil moisture conditioning, aqueous waste treatment, cable water jamming and tangible additives etc.[4]

Excessively retentive polymers are cross-connected polymer contain network like a chain without dissolving and they can retain just as grip the enormous amount of water in the swollen structure. The retained fluid is barely removable still under certain powers[5]. Super sponges have perceived critical thought as a result of their impressive applications in few zones, for example, sterile products[6-8], gardening[9], squander water treatment[10] and prescription for medication-free strategy[11]while in a wide assortment of uses, about all of the super sponges utilized as dispensable particles depend on completely oil-based polymers with high creation cost and having genuine condition sway and along these lines, they have certain limits. In water, SAPs swell to a straightforward chewy gel and their mass can suck up and hold unforeseen extraordinary measures of water or fluid arrangement[12].

The making of the primary water-spongy polymer organized from acrylic acid (AA) and the cross-connecting agent styrene was thermally polymerized in a fluid medium and afterward the main development of hydrogels was shown up[13]. These hydro gels were generally found on hydroxy alkyl methacrylate and associated monomers with swelling ability up to 50%. In the present strategy, water is utilized as a dissolvable medium and $K_2S_2O_8$ as an initiator. This strategy is useful in completing the enormous number of polymer items in lesser time when contrasted with the ordinary strategies. Water is picked as dissolvable in this polymerization response to make blend greener way.

Material and Methods

Chemicals and equipments: Allyl sucrose, styrene, acrylic acid, ethanol and $K_2S_2O_8$ are of laboratory grade. Basic glass products like three-neck round bottom flask and gear like mechanical stirrer and Brooker FT-IR spectrophotometer were used.

Synthesis of salts of cross-linked poly acrylic acid: A nitrogen gas gulf tube and mechanical stirrer were fitted with a three necked round bottom flask of 1000 ml limit. 100 ml of dissolvable blend is moved into the flask and includes two little bits of porcelain. Gradually heat the dissolvable blend to 60 °C temperature and afterward pass the nitrogen gas to sparge the flask substance to power out the air. Allyl sucrose (1, 0.8, 0.6, 0.4, 0.2 g), a diverse measure of acrylic acid and styrene are added to the response blend and mixed. In the meantime, 1 % of an initiator ($K_2S_2O_8$) is added to start the polymerization and temperature is step by step expanded to 80°C. Jellified item is seen in 45 minutes. The gelisdried for 24 hours and then granulated for further assessment. The reaction mixture is employed is as described in table 1.

Swelling test: SAP (0.5 g) samples (W_0) are placed into a pre-weighed beaker. The polymers are dipped in an excess amount of water for overnight to reach the stability of swelling. Then the excess amount of water is removed until

no liquid was dropped off. The polymers are weighed (W_1) and the swelling capacity was calculated by equation:

Swelling capacity = Wet/dry*100% = W_1/W_0*100%

Absorbance capacity of polymers is shown in table 2.

Results and Discussion

FT-IR spectra: The Fourier Transform Infrared (Brooker FT–IR spectrometer alpha Model) Spectroscopic technique is used for characterization of polymers by preparing KBr pellets of the materials. The representative IR spectrum of the polymer is displayed in figure 1. The spectra exhibit the characteristic IR peak at 3010 cm^{-1}(-C=C-H stretching) of CH_2 scissoring and the strong peak at 1696 cm^{-1} for –C=O stretching of inter-molecular hydrogen bonding of acrylic acid. The absorption band at 1188 cm^{-1} is recognized for –C - O stretching of ether bending vibration. The peak at 1395 cm^{-1} is accredited to O-H bending vibration.

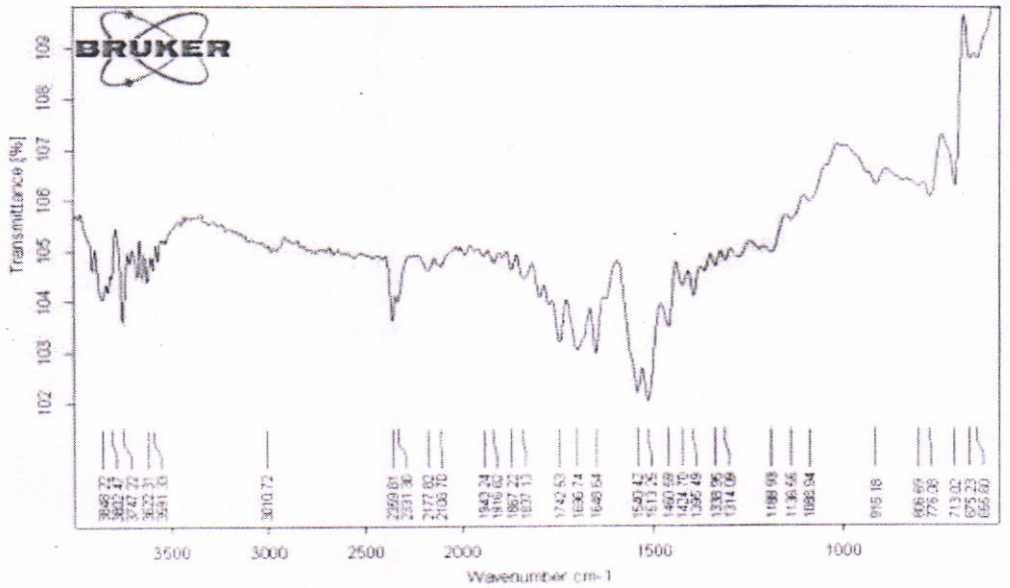


Fig. 1: FT-IR of cross-linked polymer

Table 1
Amount of reagents

CHEMICALS	AMOUNT (2.1)	AMOUNT (2.2)	AMOUNT (2.3)	AMOUNT (2.4)	AMOUNT (2.5)
Allyl sucrose	1 gm	0.8 gm	0.6 gm	0.4 gm	0.2 gm
Styrene	56 gm	56.1 gm	56.2 gm	56.3 gm	56.4 gm
Acrylic acid	43 gm	43.1 gm	43.2 gm	43.3 gm	43.4 gm
Ini.– $K_2S_2O_8$	1 gm	1 gm	1 gm	1 gm	1 gm
Sol.-H_2O+C_2H_5OH	490 + 10 ml	490 + 10 ml	490 + 10 ml	490 + 10 ml	490 + 10 ml
Neut. - NaOH	7 pH	7 pH	7 pH	7 pH	7 pH

Table 2
Absorbance capacity of polymer

Wt. of cross-linking agent	Weight of sample (g)	Weight of swelled polymer (g)	Swelling capacity %
1.0 g	0.500 g	11.760	2252.13
0.8 g	0.500 g	26.119	5123.81
0.6 g	0.500 g	1.4280	185.612
0.4 g	0.500 g	44.255	8750.97
0.2 g	0.500 g	20.903	4080.69

Thermal analysis: The thermo gravimetric analysis (TGA) of the polymers indicates (Figure 2-6) that degradation of a polymer starts around 150°C and degrades completely above 200°C to 260°C. So, the polymers are thermally stable up to 150°C. The canyons (converse peaks) are due the fact that polymer has started to degrade and heat is liberated during degradation.

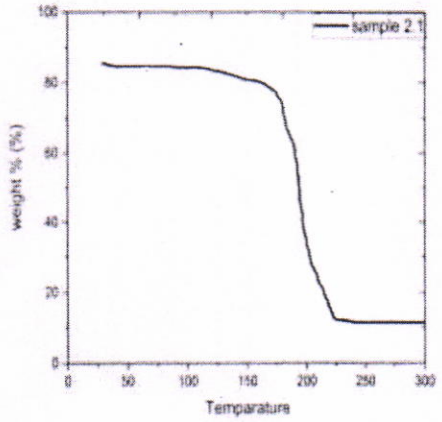


Fig. 2: TGA of cross-linked polymer (2.1)

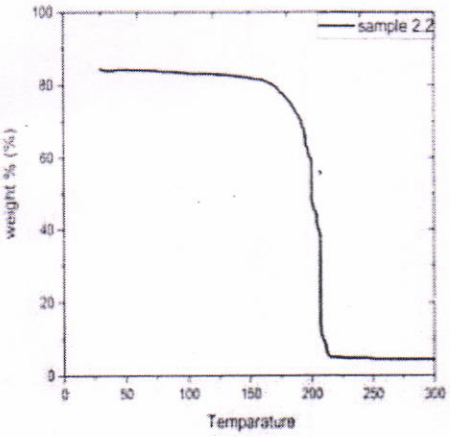
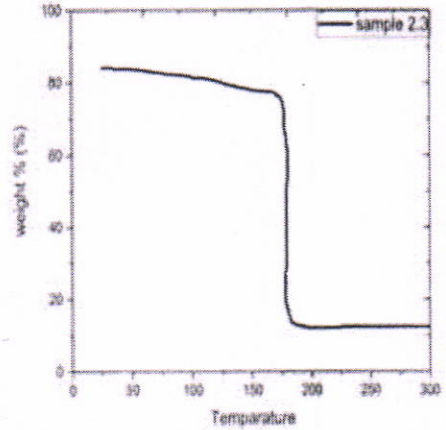


Fig. 3: TGA of cross-linked polymer (2.2)



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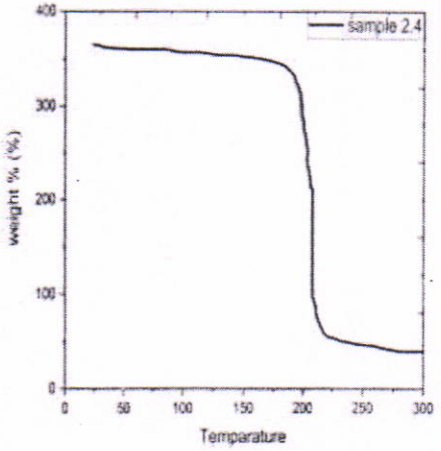
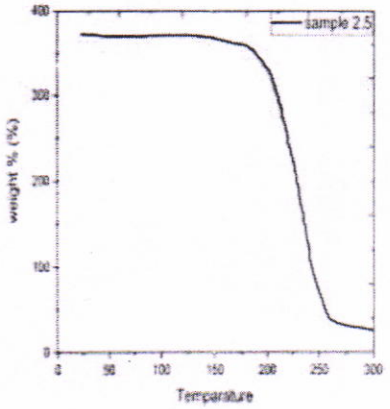


Fig. 5: TGA of cross-linked polymer (2.4)



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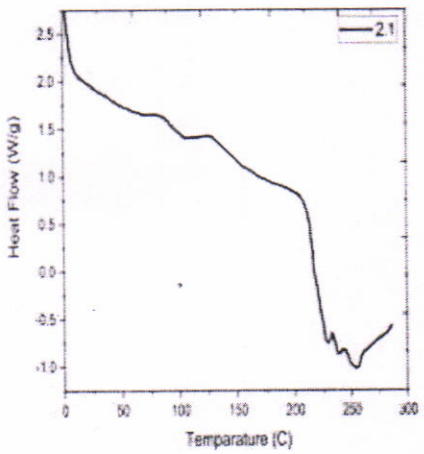

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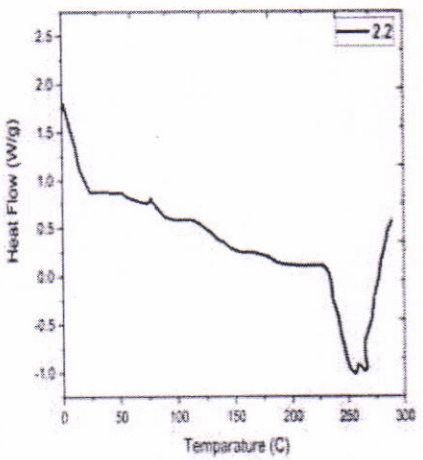


Fig. 8: DSC of cross-linked polymer (2.2)

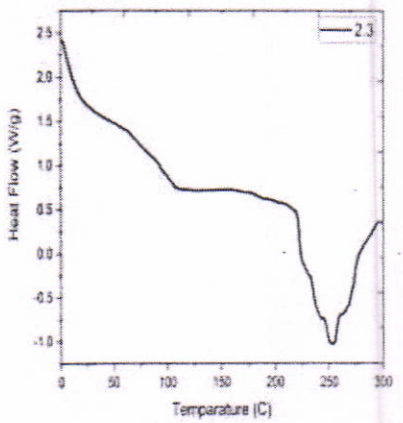


Fig. 9: DSC of cross-linked polymer (2.3)

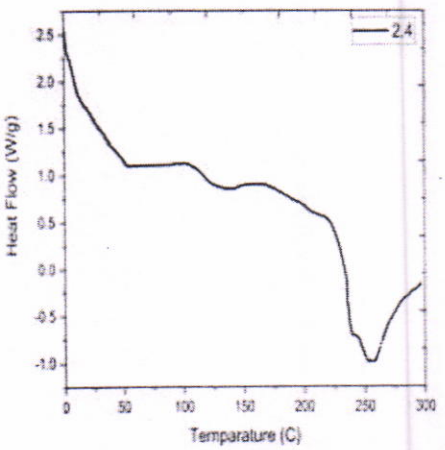
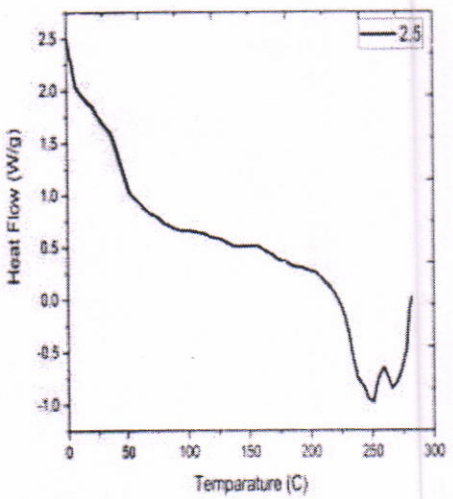


Fig. 10: DSC of cross-linked polymer (2.4)



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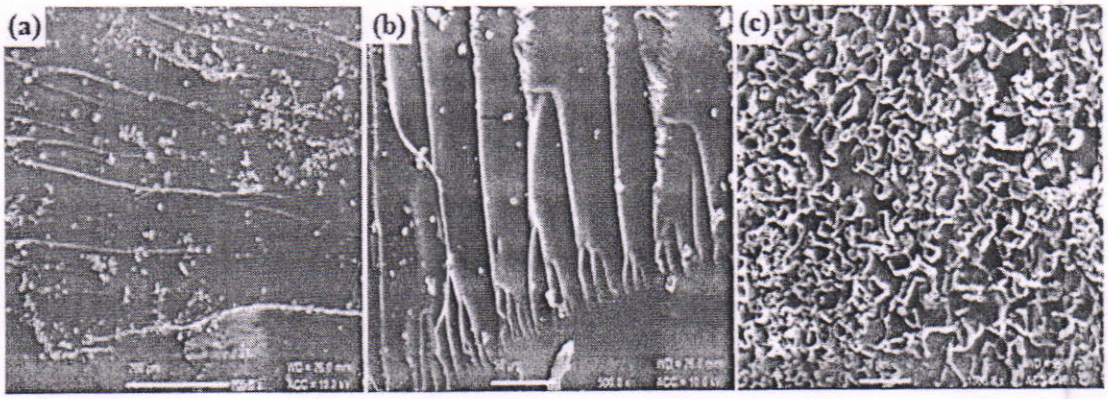

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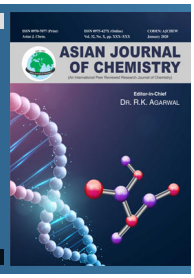
(Received 03rd September 2019, accepted 05th November 2019)

Asian Journal of Chemistry; Vol. 32, No. 12 (2020), 3127-3134

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2020.22915



Development and Validation of Modern UHPLC-DAD Analytical Method for Simultaneous Determination of Repaglinide and Metformin in Pharmaceutical Dosage Forms

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Received: 25 July 2020; *Accepted*: 5 October 2020; *Published online*: 7 December 2020; AJC-20151

For the simultaneous determination of repaglinide and metformin hydrochloride in bulk, an effective and simple UHPLC method was developed and validated and applied to marketed repaglinide and metformin products. The mobile phase used for chromatographic runs consisted of 30 mM phosphate buffer (pH 3.7) and acetonitrile (20:80, v/v) separation was implemented using isocratic mode on an Agilent Zorbax Eclipse Plus C18 (150 × 4.6 mm, 5 µm) column. Drug peaks were well separated and a 232 nm DAD detector observed them. The method was linear for repaglinide and metformin at the concentration range of 20-100 µg/mL, respectively. The method has been validated with respect to system suitability, specificity, accuracy, precision, robustness and ruggedness according to ICH guidelines. Repaglinide and metformin forced degradation studies were conducted for under acidic, base, neutral (peroxide), thermal and photo conditions.

Keywords: Repaglinide, Metformin, UHPLC, Degradation.

INTRODUCTION

Repaglinide (REPA) refers to the anti-diabetic family of meglitinides used to control type 2 diabetes [1]. Metformin hydrochloride (MET) is an anti-diabetic medication belonging to the class of biguanide [2]. Metformin hydrochloride shows its anti-diabetic action mainly by reducing the development of hepatic glucose. Meglitinides demonstrate their hypoglycemic influence by inhibiting ATP-sensitive potassium channels in the membrane of the pancreatic β-cells by inducing first-phase insulin secretion. This move is followed by a cascade of events which eventually stimulates the release of insulin from these cells decreasing the circulation of blood glucose [3]. Metformin hydrochloride is co-administered as a combined dosage form with the present drugs; subsequently, it has a different method of action to non-sulfonylurea insulin secretagogues (repaglinide, mitiglinide calcium and nateglinide), which gives a combination product more benefit than a single-component dosage form. Subsequently, the drugs cited by meglitinides are co-administered with metformin hydrochloride; the development of a technique for their simultaneous purpose with metformin hydrochloride was significant.

The metformin hydrochloride literature analysis was performed using HPLC-UV methods and alone with the column Nova-Pak silica [4], or simultaneously separation was accomplished using isocratic mode on an Alltima CN column [5]. HPLC-MS/MS approach for quantifying SGLT2 antagonists and metformin hydrochloride in plasma simultaneous and applying it to a pharmacokinetic test in fit volunteers [6]. However, the present work deals with multiple mixtures and is presenting a particular viewpoint for LC determination. As for the class of meglitinides, a few methods for their determination have been published. Metformin hydrochloride was calculated through means of spectrophotometry [7-9], HPLC [10-13] and HPTLC [14,15]. An LC method [16,17] for the simultaneous determination of repaglinide, HPTLC [18] and spectrophotometric [19,20] techniques are also reported. A variety of HPLC methods evaluating this were described for repaglinide combination with metformin hydrochloride [21-23] and even in multi-component mixtures [24,25]. The authors intend to establish appropriate HPLC methods for an immense range of medicinal products and at the similar time, appropriate for the study of counterfeits. Spectrophotometric

methods for calculating repaglinide have also been published and metformin hydrochloride binary mix [26] and HPTLC [27,28]. Most of the methods recorded for determination of repaglinide and metformin hydrochloride included plasma assay [29,30].

The present research has been effective in discussing and overcoming several problems in the assay of bulk drugs and dose forms. For instance, separation of repaglinide and metformin hydrochloride was accomplished in a short time, isocratic chromatographic sprint 6 min. Besides, under chromatographic conditions, aim and method development and validation of the method simple, precise, accurate and economic characteristics would be well illustrated further.

EXPERIMENTAL

Materials, reagents and pharmaceutical products: Repaglinide (certified to contain 99.44%) and metformin hydrochloride (certified to contain 99.10%) purchased from Clearsynth Labs Ltd. (Mumbai, India). EUREPA MF 2 (repaglinide 2 mg and metformin 500 mg tablets, Torrent Pharmaceuticals Ltd.) (India) attained from a local pharmacy. Analytical reagent grade orthophosphoric acid and potassium dihydrogen orthophosphate obtained from Finar Limited (Ahmedabad, India). HPLC grade acetonitrile and Water procured from S.D. Finechem Ltd. (India).

The UHPLC system used for the method development and validation consisted of Agilent, (CA, USA) equipped with a quaternary pump G4204A, Agilent DAD G4212A (Diode array detector), Agilent thermostat column compartment TCC G1316C and Agilent autosampler G4226A fitted with an Agilent thermostat G1330B, were used. Data acquisition, recording and chromatographic integration performed OpenLAB CDS Chem station (version A.01.05). All chromatographic evaluation and isolation conducted on the Agilent Zorbax Eclipse Plus C18 (150 × 4.6 mm, 5 µm) at 232 nm and the temperature held at 30 ºC. Phosphate buffer (4 g), pH 3.7 and acetonitrile at ratio (20:80, v/v), use as a mobile phase, a flow rate of 1 mL/min in isocratic mode and an injection volume of 5 µL for all.

Preparation of buffer solution: Buffer solution the concluding is collected of 4.0 g KH_2PO_4 in 1000 mL HPLC water, pH adjusted to 3.7 by orthophosphoric acid. Buffer solution filtered through (0.45 µ nylon membrane filter) and degassed for 20 min in a sonicator.

Preparation of standard stock solutions: Accurately weighed 50 mg of repaglinide and metformin hydrochloride were separately transferred into 100 mL volumetric flasks and dissolved in 70 mL of the mobile phase mixture sonicated for 20 min. The final volume made up of the mark with the mobile phase mixture.

Preparation of working solution: Accurately calculated 1 mL aliquots from standard stock solutions were moved to volumetric flasks of 10 mL and finally completed to volume, utilizing REPA and MET as standard working solutions (50 mg/mL).

EUREPA MF 2 tablets, preparation: Twenty tablets EUREPA MF 2, claimed to contain 2 mg REPA and 500 mg MET were weighed precisely and then powder using a mortar pestle and fine particle size. The exact weight of this powder equivalent to the one tablet content was considered, transferred to a volumetric flask of 20 mL, added 10 mL of the mobile phase, sonicated for about 40 min and then completed with the same mobile phase to the volume. This solution (10 mL) was transferred to 20 mL volumetric flasks made up to the mobile phase mark and an additional 0.4 mL aliquot from Flask was transferred to 100 mL volumetric flasks. The mobile phase was added to the mark and filtered (Hydrophilic PVDF 0.22 µm) to produce a final concentration of 50 µg/mL repaglinide and 50 µg/mL metformin hydrochloride, respectively.

Method development and optimization: Method development and optimization certain mobile phases and columns initially checked to have all eluents on the same chromatogram due to the significant difference in chemical and physical properties of repaglinide and metformin hydrochloride. Based on the specificity, selectivity and correct chromatographic parameters of the formed peaks calculated in terms of peak symmetry, peak sharpness, resolution and tailing factor between the two peaks, column suitability and the used as a mobile phase in the improved method. We used the solvent as a mobile phase for all samples to confirm the minimum noise and eliminate any inappropriate solvent peaks.

Columns applied in our initial trials: Efforts were made by using four kinds of UHPLC columns Zorbax Eclipse Plus C18 (50 × 4.6 mm, 1.8 µm), Inertsil ODS-2 (150 × 4.6 mm, 5 µm), Zorbax Eclipse Plus C18 (150 × 4.6 mm, 5 µm), Phenomenex Synergi C18 (150 mm × 4.6 mm × 4 µm) for the improved method.

Types of buffers and different mobile phase evaluated using: Various concentrations of phosphate buffer with (20, 30, 40, 50 and 60 mM) then used to increase the polarity of the mobile phase resulting in a narrowed peak.

Several mixture solutions with various pH levels (2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) were used to analyze the retention time and resolution of repaglinide and metformin hydrochloride under which the other chromatographic parameters were kept unchanged. A 30 mM and 3.7 pH phosphate buffer for the optimized method.

Selection of UV wavelength: Repaglinide has a λ_{max} at 241 nm and metformin hydrochloride has λ_{max} at 234 nm. The acceptable response obtained when both drugs were detected at 241 nm either independently or in combination.

Method validation: The standardized procedure for the simultaneous assessment of repaglinide and metformin hydrochloride was tested in accordance with the International Conference on Harmonization (ICH) Guidelines Q2 (R1) [32, 32] for the evaluation of limit of detection (LOD), limit of quantitation (LOQ), system suitability, precision, accuracy, specificity, robustness and ruggedness.

System suitability: Six replicates injections also determined the system's suitability repaglinide and metformin hydrochloride (50 µg/mL). The method developed was found suitable for use as tailing factor, repeatability, number of theoretical plates repaglinide and metformin hydrochloride resolution was within limits.

Precision, repeatability (intra-day precision) and intermediate precision (inter-day precision): The precision of the system and the method were tested by injecting six separate combinations of repaglinide and metformin hydrochloride samples (50 µg/mL) on the same day beneath the same operating conditions. Intermediate or inter-day precision was analyzed by evaluating the effects on three different days of six independent determinations.

Linearity and range: The standard repaglinide and metformin hydrochloride stock solution are diluted within the concentration range of (20-100 µg/mL). Triplicates of this concentration range were prepared and plotted on a calibration curve for repaglinide and metformin hydrochloride. This concentration range had been developed and plotted on a calibration curve for repaglinide and metformin hydrochloride. To ensure the linearity of the analytical method, the slope, intercept and correlation coefficient of the calibration curves (peak area *versus* concentration) was defined.

Accuracy study and recovery: The accuracy of the suggested method was verified by the placebo spiking process, which was achieved separately by spiking placebo with repaglinide and metformin hydrochloride at three different levels, 80%, 100% and 120%. Triplicate assessments of these three levels to determine the mean and % RSD were reported.

Method sensitivity, LOD and LOQ: LOD and LOQ for repaglinide and metformin hydrochloride were determined based on the linear regression equation:

$$LOD = 3.3 \times \frac{\text{Standard deviation (of response)}}{\text{Slope of calibration curve}}$$

$$LOQ = 10 \times \frac{\text{Standard deviation (of response)}}{\text{Slope of calibration curve}}$$

Robustness and ruggedness: Intentional minute changes were made in the chromatographic conditions such as wavelength, flow rate, temperature and pH of the buffer. Such differences were also tested for resolution between peaks of repaglinide and metformin hydrochloride, retention time, number of theoretical plates, asymmetric factor and % RSD.

Forced degradation study

Acid degradation: Forced degradation of repaglinide and metformin hydrochloride (50 µg/mL) by acid hydrolysis using 1 M HCl maintained for 2 h at 60 ºC. The sample was applied to the target after the stress was neutralized with NaOH and diluted with the mobile phase and filtered (Hydrophilic PVDF 0.22 µm) before the study.

Base degradation: Forced degradation of repaglinide and metformin hydrochloride (50 µg/mL) by base hydrolysis using 1 M NaOH maintained for 2 h at 60 ºC. The sample was applied to the target after the stress was neutralized with hydrochloric acid and diluted with the mobile phase and filtered (Hydrophilic PVDF 0.22 µm) before the study.

Hydrogen peroxide (neutral) degradation: Forced degradation of repaglinide and metformin hydrochloride (50 µg/mL) was observed under the impact of (3%) H_2O_2 maintained for 2 h at 60 ºC. The stressed sample was diluted with mobile phase and filtered (Hydrophilic PVDF 0.22 µm) before the study.

Thermolysis degradation: The effect of rising temperature on REPA and MET (50 µg/mL) was observed by heating the sample in refluxing apparatus at 60 °C for 48 h. The stressed sample was diluted with mobile phase and filtered (Hydrophilic PVDF 0.22 µm) before the study.

Photolytic degradation: The effect of UV light on the REPA and MET (50 µg/mL) stability was analyzed by 48 h illumination of the sample in UV light at 365 nm. The stressed sample was diluted with mobile phase and filtered (Hydrophilic PVDF 0.22 µm) before the study.

RESULTS AND DISCUSSION

Method development and optimization: Our goal in the present research was to establish a quick, inexpensive, selective and responsive system for the simultaneous determination of the antidiabetic class of meglitinides with repaglinide and metformin hydrochloride. Developing a sufficiently precise analytical method for discriminating against repaglinide and metformin hydrochloride has been highly challenging. Simple, HPLC with DAD detection was used to do this. Preliminary tests on C18 column at ambient temperature achieved separation between repaglinide and metformin hydrochloride Nevertheless, further optimization of chromatographic conditions was required to achieve separation of the two target products from the meglitinides along with demonstration of appropriate peak shape, selectivity, sensitivity and fulfillment of all other system suitability parameters. A study was performed to explain the impact of each parameter on component separation and sufficient elution to achieve the optimum conditions needed for the separation of target products; a buffer component of 30 mM potassium dihydrogen orthophosphate was measured at various pH values (2.5, 3.0, 3.5, 4.0 and 5.0); acetonitrile was observed at varies concentrations (20:80, 40:60, 60:40 and 80:20) and at different flow rates (0.8, 0.9, 1 and 1.2) were assessed.

Through referring to the selection step in the test, the optimum separation was obtained using a 30 °C and C18 column using a mobile phase consisting of 20% 30 mM potassium dihydrogen orthophosphate (pH adjusted by orthophosphoric acid to 3.7): 80% acetonitrile which flows at a rate of 1.0 mL/min. In a run time of 6 min, eluted peaks were observed at 232 nm. Deficiency of separation between repaglinide and metformin hydrochloride did not pose an incorrect outcome or undesirable matter as the two components fit the similar class of antidiabetics, which stops any option of uniting them in a single dosage type. Hence, their coordinated separation is not necessary. For repaglinide and metformin hydrochloride, respectively, retention periods (min) obtained under acceptable chromatographic conditions were 3.72 and 1.33 (Fig. 1). The requirements of the method, therefore, specified earlier under chromatographic conditions, are considered suitable to separate in the presence of the two oral antidiabetic drugs.

System suitability: The results obtained from six replication injections suggested that the parameters evaluated were within the appropriate range. Repaglinide and metformin hydrochloride were maintained consistently and well separated at

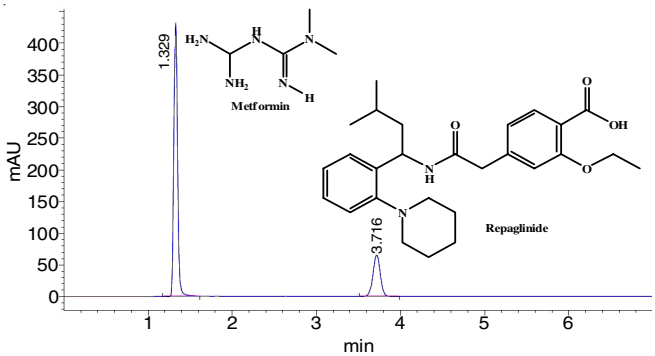

Fig. 1. UHPLC chromatogram of repaglinide and metformin hydrochloride. Chromatographic conditions: Zorbax Eclipse Plus C-18 (150 mm × 4.6 mm i.d., particle size 5 µm); mobile phase phosphate buffer (30 mM potassium dihydrogen phosphate, pH adjusted to 3.7 ± 0.02 with orthophosphoric acid) and acetonitrile (20:80 v/v); flow rate of 1.0 mL/min; and DAD detection at 232 nm

3.7 min and 1.3 min respectively, demonstrating excellent resolution of both peaks with an % RSD of the reported retention periods < 0.3 to suggest stable repeatability of replicate injections on the integrated UHPLC system used, the tailing factor for both repaglinide and metformin hydrochloride peaks never reached 1.0 in both peaks suggesting good peak symmetry (acceptance limit is < 2). The number of theoretical plates in all chromatographic runs was always > 2000 to ensure good column efficacy during the separation cycle established. Results are displayed in Table-1.

TABLE-1
SYSTEM SUITABILITY RESULTS FOR REPA AND MET

Parameters	REPA	MET
Peak area (A) mAs	433.30 ± 0.96	1565.50 ± 3.01
Relative standard deviation (RSD)	0.22%	0.19%
Retention time (tR)	3.72	1.33
Theoretical plates (N)	6949	3921
Symmetry factor (AS)	0.99	0.98
Resolution	10.61	–
Retention factor K'	6.46	1.66

Precision: The peak areas obtained after injecting six individual combined samples of repaglinide and metformin hydrochloride were repeatable and consistent for two consecutive days. The findings for both intra-day and inter-day determinations maintain the high precision and repeatability of the constructed system where all data was presented in % RSD and never surpassed 0.53 % (% RSD < 2 approval limit). Results for intra-day and inter-day precision displayed in Table-2.

TABLE-2
INTRA-DAY AND INTER-DAY PRECISION RESULTS

Analysis date	Intra-day		Inter-day	
	REPA	MET	REPA	MET
% Assay Mean	99.78	99.90	100.42	99.88
% RSD	0.53	0.29	0.37	0.52

Specificity: The analytical method was capable of detecting and assessing repaglinide and metformin hydrochloride in the presence of a typical tablet excipient matrix. The representative chromatogram of mobile phase, placebo and repaglinide and metformin hydrochloride, standard mixture, is displayed in Fig. 2. The specificity of the system was verified when the optimized conditions for detecting repaglinide and metformin hydrochloride (from manufacturer's excipients) were implemented in EREPA MF 2 tablets, respectively, representative repaglinide peak and metformin hydrochloride peak analysis of marketed tablets displayed in Table-3, while chromatograms in tablets are displayed in Fig. 3.

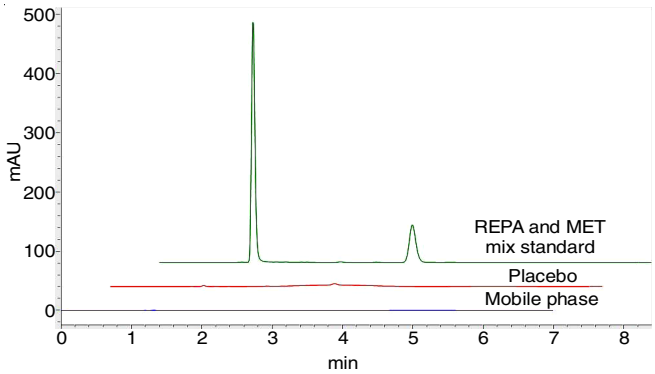

Fig. 2. Overlaid chromatograms of mobile phase, placebo and repaglinide and metformin hydrochloride standard mixture

Linearity: In the specified ranges, the analytical calibration curve constructed for both repaglinide and metformin hydrochloride was linear, indicated by the closeness of the correlation coefficient R^2 to 1 ($R^2 = 0.9999$). The linear regression equation for repaglinide is ($y = 8.174x - 3.810$, $R^2 = 0.999$) and the linear

TABLE-3
STUDY OF MARKETED TABLETS

Tablet (EREPA MF 2) Replicate number	Retention time		Area		Symmetric factor		Number of theoretical plates		Assay (%)	
	REPA	MET	REPA	MET	REPA	MET	REPA	MET	REPA	MET
1	3.718	1.326	434	1562	0.99	0.98	6924	3931	100.38	99.78
2	3.719	1.326	434	1566	0.99	0.98	6920	3921	99.58	99.69
3	3.719	1.327	434	1568	0.99	0.98	6981	3911	99.38	100.23
4	3.718	1.326	432	1565	0.99	0.96	6949	3931	100.07	99.54
5	3.719	1.326	433	1563	0.99	0.97	6973	3921	100.38	100.48
6	3.718	1.327	432	1570	0.99	0.98	6949	3911	100.81	99.63
Mean ± SD	3.719 ± 0.00	1.326 ± 0.00	433 ± 0.96	1565 ± 3.01	0.99 ± 0.00	0.97 ± 0.01	6949 ± 24.76	3921 ± 8.94	100.09 ± 0.54	99.89 ± 0.37
%RSD	0.01	0.04	0.22	0.19	0.00	0.86	0.36	0.23	0.54	0.37

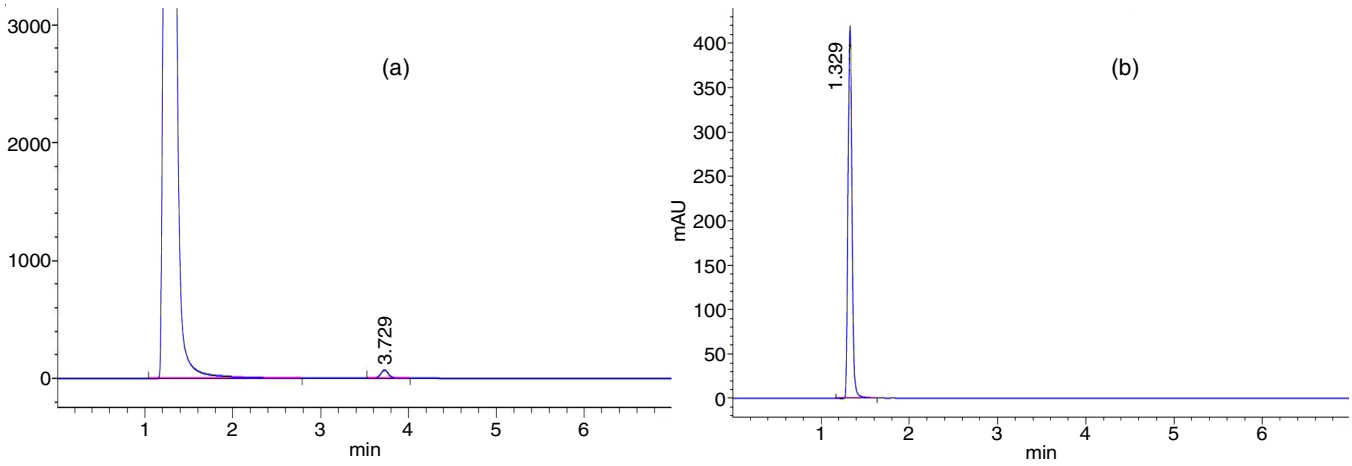

Fig. 3. UHPLC chromatogram of EREPA MF 2 capsule sample solution containing (a) 50 µg/mL repaglinide and (b) 50 µg/mL metformin hydrochloride

regression equation for metformin hydrochloride is ($y = 21.73x + 42.71$, $R^2 = 0.999$) are displayed in Tables 4 and 5. The calibration curves that were produced by plotting peak area against concentration displayed linear relation. Calibration curves with corresponding residual plots repaglinide and metformin hydrochloride are displayed in Fig. 4.

TABLE-4
LINEARITY RESULTS OF REPA AND MET

STD Concentration range (µg/mL)	Peak area		Found concentration	
	REPA	MET	REPA	MET
20	159	467	19.23	19.08
30	243	677	29.48	28.84
40	326	912	39.80	39.76
50	395	1155	48.29	51.02
60	490	1351	59.94	60.16
70	568	1571	69.65	70.39
80	655	1804	80.33	81.19
90	734	1989	90.14	89.78
100	809	2192	99.36	99.21

TABLE-5
LINEARITY PARAMETERS FOR THE REPA AND MET

Linearity parameter	REPA	MET
Range (µg/mL)	20-100	20-100
Slope	8.17	21.73
Intercept	3.81	42.71
Regression coefficient (r^2)	0.999	0.999
Standard error of Intercept	4.07	15.34
Standard deviation of intercept	12.20	46.02
Confidence limit of the slope	8.17 ± 0.78	21.73 ± 0.97
Confidence limit of the intercept	3.81 ± 3.83	42.71 ± 8.42

Recovery: The accuracy of the experimental, analytical method was tested by evaluating the added analytes in the placebo matrix in triplicates at three separate levels (80, 100 and 120%) and represented in terms of percentage recovery from the spiked form of repaglinide and metformin hydrochloride. The similarity of the observed analyte values to the theoretical concentrations reported at various rates demonstrated the trueness/accuracy of the proposed method where repaglinide and metformin hydrochloride > 99% recovered from the spiked excipients. Details for recoveries from repaglinide and metformin hydrochloride are displayed in Table-6.

TABLE-6
PERCENT RECOVERY RESULTS REPA AND MET

Drug	Simulated dosage nominal (%)	% Mean (n = 3)	RSD (%)	RE%
REPA	50	100.02 ± 0.70	0.7	0.02
MET	50	100.19 ± 0.64	0.64	0.19
REPA	100	99.96 ± 0.27	0.27	-0.04
MET	100	100.10 ± 0.22	0.22	0.1
REPA	150	99.65 ± 0.75	0.75	-0.35
MET	150	99.83 ± 0.40	0.4	-0.17

LOD and LOQ: The calculated LOD and LOQ were 1.64 mg/mL, 4.93 mg/mL for repaglinide and 2.33 mg/mL, 7.06 mg/mL for metformin hydrochloride, respectively are displayed in Table-7.

TABLE-7
VALUES OF LOD AND LOQ

Drug	LOD (µg/mL)	LOQ (µg/mL)
REPA	1.64	4.93
MET	2.33	7.06

Robustness and ruggedness: No significant adjustments observed when adding minor variations to the chromatographic conditions ensuring the method is robust to small intentional modifications introduced in terms of the wavelength, flow rate, temperature, pH of the buffer used. Increasing the previous parameters was modified, thus holding the other chromatographic system parameters unchanged. Retention time, theoretical plates, symmetric factors have not changed significantly by adding the various conditions maintaining the robustness of the method described. The retention time, theoretical plates, symmetric factors under different conditions are summarized in Table-8.

Forced degradation study: Repaglinide and metformin hydrochloride were pressured under different conditions and UHPLC was subject to separation of the samples. Significant drug degradation peaks were observed under basic and neutral

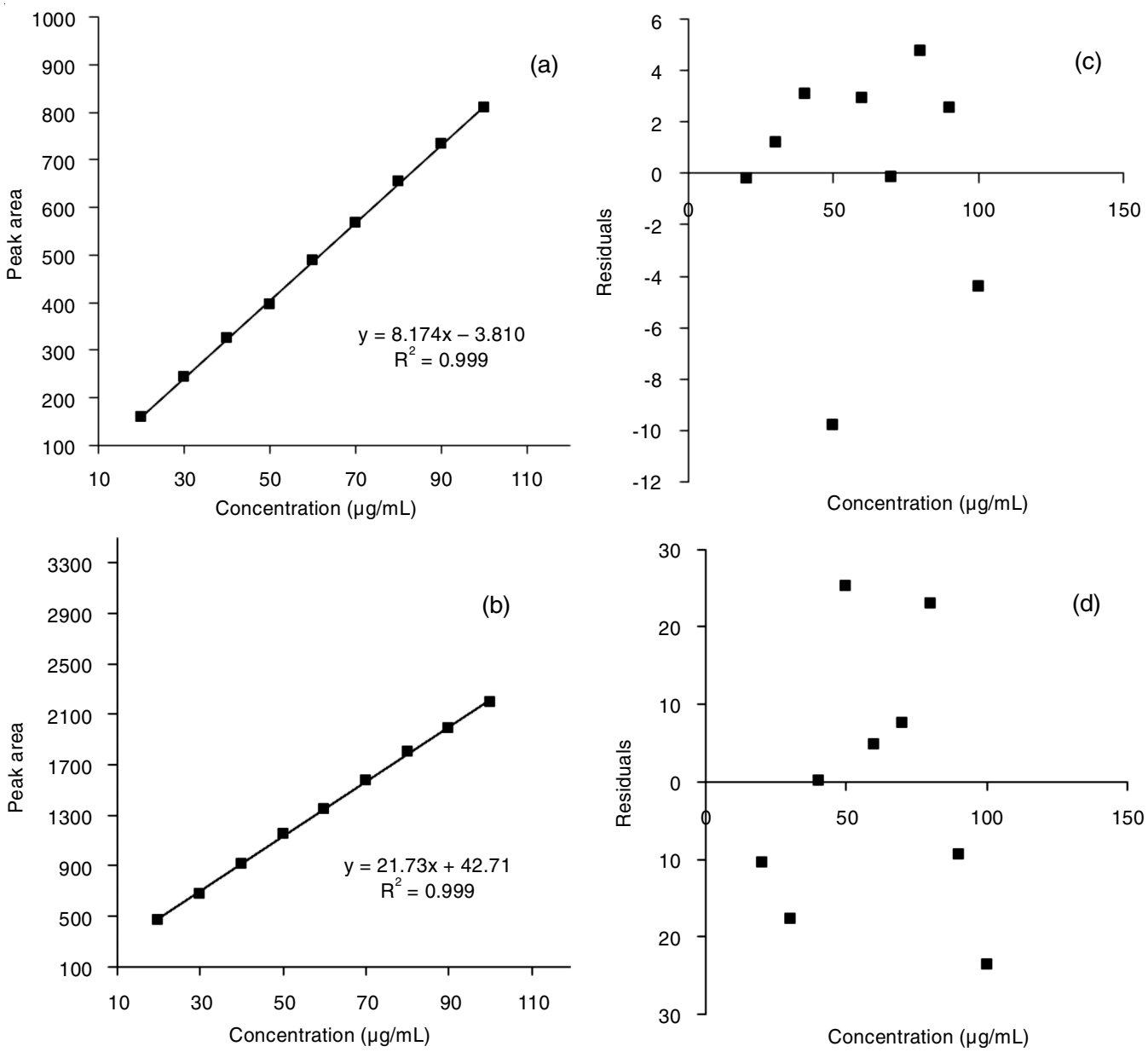

Fig. 4. Linearity plots for repaglinide (a) and metformin hydrochloride (b) with corresponding residual plots for the repaglinide (c) and metformin hydrochloride (d)

TABLE-8
ROBUSTNESS AND RUGGEDNESS RESULTS OF REPAGLINIDE (REPA) AND METFORMIN HYDROCHLORIDE (MET)

Parameter	Conditions	% RSD (n = 3)		Retention time		Number of theoretical plates		Symmetric factor	
		REPA	MET	REPA	MET	REPA	MET	REPA	MET
Change in λ_{max} 241 ± 2	239	0.15	0.3	3.61	1.34	7560	4985	0.99	0.97
	243	0.73	0.19	3.62	1.35	7257	4978	0.99	0.96
Change in flow rate 1 ± 2	0.8	0.58	0.25	4.51	1.68	8938	6783	0.99	0.98
	1.2	0.87	0.46	3	1.12	5887	3946	0.97	0.99
Change in temp. 30 ± 5	25	0.32	0.29	3.73	1.34	7187	5149	0.91	0.93
	35	0.2	0.19	3.52	1.34	7135	4893	0.99	0.97
Change in pH 3.7 ± 2	3.68	0.39	0.33	3.61	1.33	7828	5243	0.96	0.98
	3.72	0.33	0.23	3.84	1.35	7916	5276	0.94	0.98
Ruggedness									
Different analyst	Analyst 1	0.18	0.21	3.72	1.33	6942	3921	0.99	0.98
	Analyst 1	0.57	0.32	3.72	1.33	6957	3924	0.99	0.97

(H_2O_2) conditions. Lastly, the terms of acid, light and thermal stress did not encourage the formation of degradation products. The chromatograms of pure drugs and their stressed samples were seen in Fig. 5b-f. Table-9 reported peak retention time, repaglinide and metformin hydrochloride recovery percentage degradation under different stress conditions.

Conclusion

For the simultaneous separation and quantification of repaglinide and metformin hydrochloride in bulk, laboratory-prepared mixture and pharmaceutical preparations, the suggested UHPLC approach has the advantages of simplicity, precision, accuracy and convenience. The proposed UHPLC approach can,

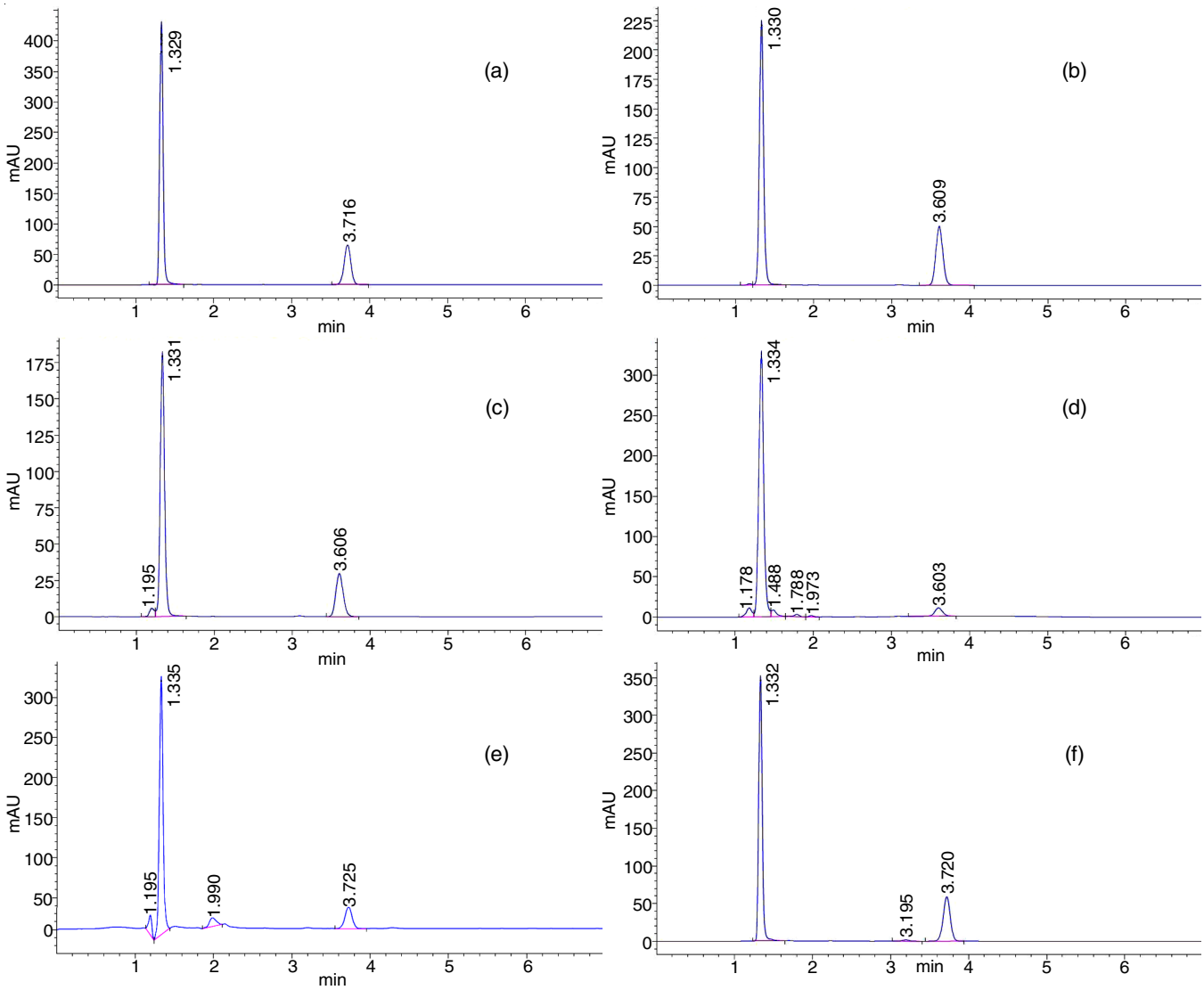

Fig. 5. (a) UHPLC chromatogram of a standard sample solution containing REPA and MET. UHPLC chromatogram of REPA and MET obtained from degradation studies, (b) Acid hydrolysis (1 M HCl at 60 °C for 1 h); (c) Base hydrolysis (1 M NaOH at 60 °C for 1 h), (d) Oxidative degradation (3% H_2O_2 at 80°C for 1 h), (e) Thermal degradation (60 °C for 48 h), (f) Photo degradation at 25°C for 48 h with UV radiation at 365 nm)

TABLE-9
DEGRADATION STUDY OF REPAGLINIDE AND METFORMIN HYDROCHLORIDE

Condition	Repaglinide				Metformin hydrochloride			
	Rt	Recovery ± SD	%RSD	% Drug degraded	Rt	Recovery ± SD	%RSD	% Drug degraded
Acid hydrolysis	3.62	84.89 ± 0.35	0.41	14.67	1.33	98.56 ± 0.50	0.51	1.15
Base hydrolysis	3.62	74.43 ± 0.58	0.77	24.78	1.33	75.79 ± 0.44	0.58	22.99
Oxidative degradation	3.61	70.13 ± 0.65	0.93	28.83	1.33	91.97 ± 0.63	0.69	6.95
Thermal degradation	3.72	91.83 ± 0.42	0.85	7.92	1.33	93.85 ± 0.61	0.65	5.47
Photo degradation	3.72	96.49 ± 0.35	0.36	3.17	1.33	98.63 ± 0.43	0.43	0.96

therefore, be used to control the quality of the above drugs with sufficient selectivity and efficiency in a short time and with low solvent usage. Such elements have been identified to see the requirements of the chromatographers for pharmaceutical drug quality evaluation.

ACKNOWLEDGEMENTS

The authors are grateful thank Pramukh Swami Science & H.D. Patel Arts College, Kadi and Clearsynth Labs Ltd. for providing samples of REPA and MET.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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RJC

RASĀYAN *J. Chem.*
Vol. 13 | No. 1 |333 - 338| January - March | 2020
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

SYNTHESIS AND CHARACTERIZATION OF CROSS-LINKED TRI-POLYMERS OF POLY ACRYLIC ACID AS WATER THICKENING AGENTS

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ABSTRACT

A cross-linked poly acrylic acid tri-polymer is successfully synthesized by batch charging polymerization of poly allyl tris buffer, di-vinyl benzene and acrylic acid and this new method is profitable in terms of reducing the toxicity, faster, purity and cheaper than available methods. The morphological structure of the synthesized polymer is studied by SEM and the characteristic functional groups of the synthesized polymers are identified using FTIR. TGA and DSC techniques were employed to study their thermal characterization.

Keywords: Poly allyl tris buffer, Di-vinyl Benzene, Acrylic Acid, Water-absorbing Agent, Copolymer Composition

INTRODUCTION

Co-polymers of the carboxylic monomer for instance acrylic acid and 0.1 - 1.0 by weight cross-linker like poly allyl tris buffer are gel-like polymers. These types of polymers are unsolvable in aqueous medium and other organic solvents. They are particularly in the type of their salts; suck up great quantities of water or other solvents with subsequent a numerous times raise in volume. The carboxylic polymers produced when a carboxylic monomer is copolymerized with a poly alkenyl polyether of a polyhydric alcohol. It is containing two or more alkenyl ether grouping per molecule.[1-2]

These carboxylic polymers are used in various fields such as thickeners, suspending agents and stabilizers.[3-6] Polymers of a carboxylic acid monomer and acrylic esters having aliphatic long-chain containing at least 10 - 30 carbon atoms can be used as thickeners when they are neutralized. They have superior resistance to reducing in viscosity when salt added thereto.[7]

Excessively spongy polymers are crossly linked polymer have a network like a sequence without dissolving and can retain just as grasp the vast volume of water in the swollen arrangement. The retained fluid is scarcely removable still under certain powers.[8]

The most frequently working synthetic polymers are evenly cross-linked carboxylic polymers primed from unsaturated carboxylic acid-containing monomers such as acrylic acid and maleic acid and anhydride. The cross-linked with unsaturated di-esters, di-vinyl benzene and others of carboxylic monomers are difficult to control in this polymerization. The various processes for synthesizing the higher carboxylic polymers are described in different Patents.[9-11] pH-sensitive cross-linked AA/Gelatin hydrogels produced by free-radical polymerization in the presence of ammonium persulfate initiator and ethylene glycol di methacrylate cross-linking agent. Different feed ratios of acrylic acid, gelatin, and EGDMA were used to investigate the effect of monomer, polymer, and degree of cross-linking on swelling and release pattern of the model drug.[13] Zinc oxide (ZnO) Nano composite hydrogels are

http://dx.doi.org/10.31788/RJC.2020.1315526

synthesized with free-radical polymerization in methanol using ammonium persulphate as the initiator at 60 ºC temperature and N, N'- methylene bis acrylamide (MBA) as a crosslinker.[14] The high swelling polymer hydrogel from epichlorohydrin with hydrolyzed polyacrylonitrile is synthesized and the swelling properties are identified. The effect of temperature and concentration is also analyzed. [15] Gold nanoparticles are prepared with poly (acrylic acid) and cysteine, it is characterized by techniques. [16]
The present research studies have employed a new synthetic method for the preparation of the tri polymers of AA.

EXPERIMENTAL

For this research work allyl tris buffer, di-vinyl benzene, acrylic acid, ethanol and potassium thiosulphate all these reagents are of laboratory reagent grade. Basic glassware such as three-neck round bottom flask and mechanical stirrer and FT-IR spectrophotometer (Bruker alpha) were used.
A nitrogen gas inlet tube and mechanical stirrer are built-in with a three-necked round bottom flask of 1000 ml capacity. Exactly 600 ml of the solvent mixture is transferred into the flask and add the two small pieces of porcelain chips. Now gradually heat the solvent mixture to 60-65ºC temperature and then pass the nitrogen gas to sparge the flask contents to oust the air. The 5 experimental runs (3.1-3.5) are set using varied amounts of allyl tris buffer (1, 0.8, 0.6, 0.4, 0.2 g) and relevant of acrylic acid and di-vinyl benzene added. At the same time, 1 g of an initiator potassium thiosulphate ($K_2S_2O_8$) is added to initiate the polymerization and temperature is expanded by 4°C/min to reach 80°C and this temperature is maintained for 1.5 h. During this heating period, the gelatinized polymer starts to produce within 50 minutes. The produced strong gel is dried for 24 hours. The dried solid is then used for further investigation. Then weigh 0.5 g solid polymer in a beaker to make suspense solution in 30 ml water overnight. Then remove the excess amount of water from a beaker and the swollen product is neutralized with saturated NaOH at about 7.0 pH. The reaction mixtures employed are given in Table-1.

Table-1: Description of the Amount of Reagents

Experiment	3.1	3.2	3.3	3.4	3.5
Chemicals	Amount	Amount	Amount	Amount	Amount
Allyl tris buffer	1 g	0.8 g	0.6 g	0.4 g	0.2 g
Di-vinyl benzene	39 g	39.1 g	39.2 g	39.3 g	39.4 g
Acrylic acid	60 g	60.1 g	60.2 g	60.3 g	60.4 g
Ini.– $K_2S_2O_8$	1 g	1 g	1 g	1 g	1 g
Sol.-H_2O+C_2H_5OH	490 + 10 ml	490 + 10 ml	490 + 10 ml	490 + 10 ml	490 + 10 ml
Neut. - NaOH	7 pH	7 pH	7 pH	7 pH	7 pH

RESULTS AND DISCUSSION

The polymer thickeners are identified by Fourier Transform Infrared (BROOKER FT-IR) Spectrophotometer using their KBr pellets and the representative IR spectrum of the polymer 3.1 is presented in Fig.-1. The spectra show that the main characteristic peaks are at 2924 $cm^{-1}$and 2855 cm^{-1}. These are exhibited for the asymmetric stretching vibration of (-C=C-H Stretching) and asymmetric stretching vibration of N-H of the secondary amine group. The strong peak at 1696 cm^{-1} confirmed –C=O stretching of intermolecular hydrogen bonding of acrylic acid. Furthermore, the IR band at 1652 (merged, weak) cm^{-1} is owing to the characteristic stretching vibration of -C=C bond. The peak at 1396 cm^{-1} is accredited to O-H bending vibration. The band at 1156 cm^{-1} is recognized for –C - O stretching of ether bending vibration.
The thermo gravimetric analysis (TGA) of the polymers was run using TG (Perkin Elmer TGA 8000 Thermo Gravimetric Analyzer). The thermograms indicate that degradation of a polymer starts around 150°C and degradation completed above 200°C temperature. Thus, it can be concluded that the prepared polymer thickeners are thermally stable up to 150°C and few are up to 250°C. The canyons (converse peaks) are due to the fact that polymer has started to degrade as the impurity could be adhered in the form of aqueous phase or hydrocarbons and heat is liberated during degradation.
The cross-linking is also characterized by DSC analysis, as shown in Fig.-3. DSC of cross-linked thickener polymer indicates that a weight loss occurs within two stages.

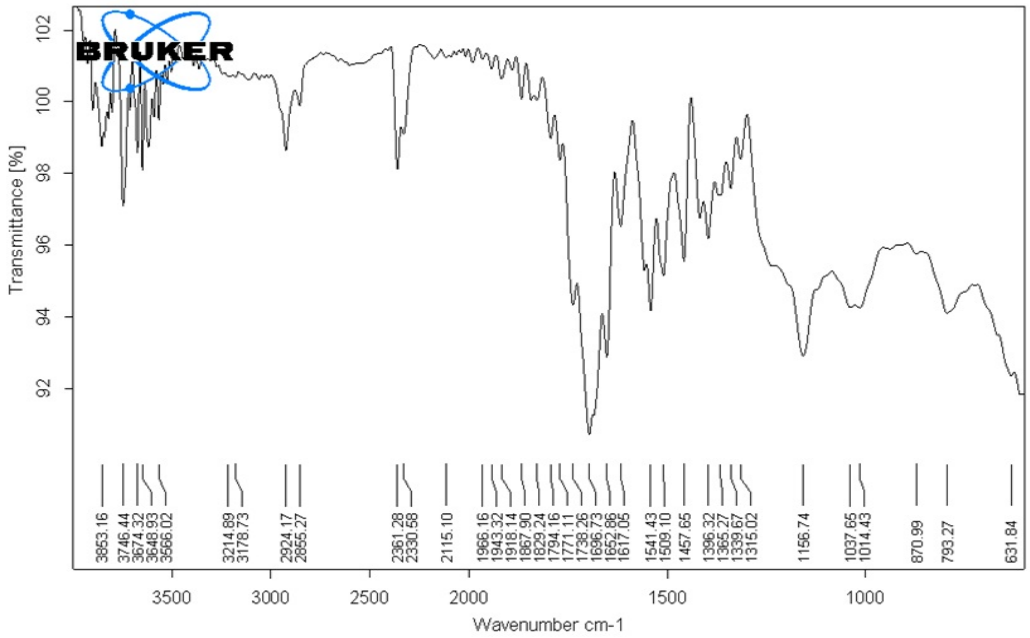

Fig.-1: FT-IR of Cross-linked Tri-polymer

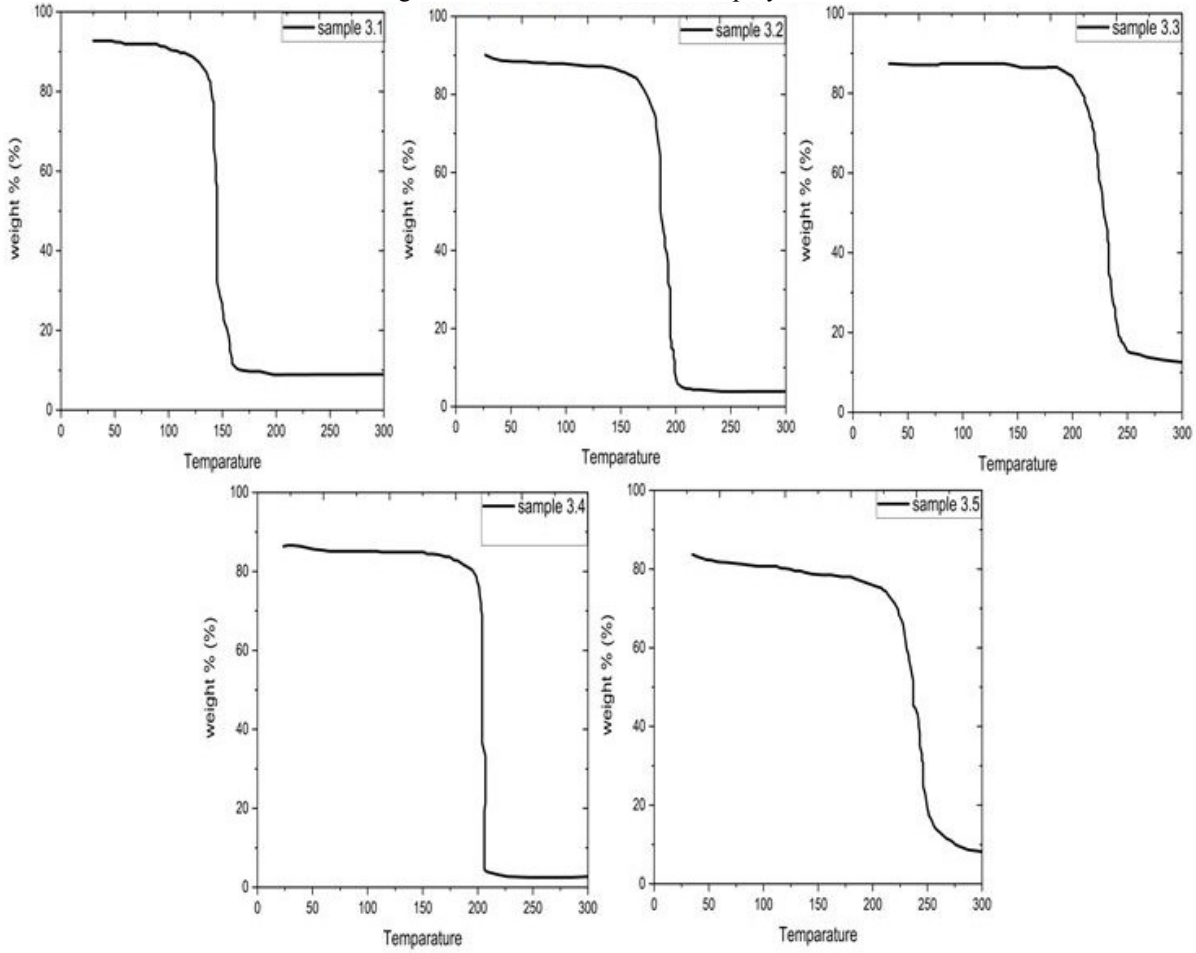

Fig.-2: TGA of Cross-linked Tri-polymers

Essentially, acrylic acid has the glass-transition temperature *(Tg)* is nearly 106°C, but due to cross-linked with DVB, there may be slightly changed in T_g. DSC thermograms designate that T_g of the polymer increases by about 115°C and is independent of the amount of allyl tris buffer. There is the hump in the bend and fix the midpoint in each curve as T_g. The initial stage shows weight loss is between 40°C and 125°C which might be due to the loss of absorbed and bound water. The next stage of mass loss started at 225°C and continued to 275°C, during which weight loss may be due to the degradation of the cross-linker.

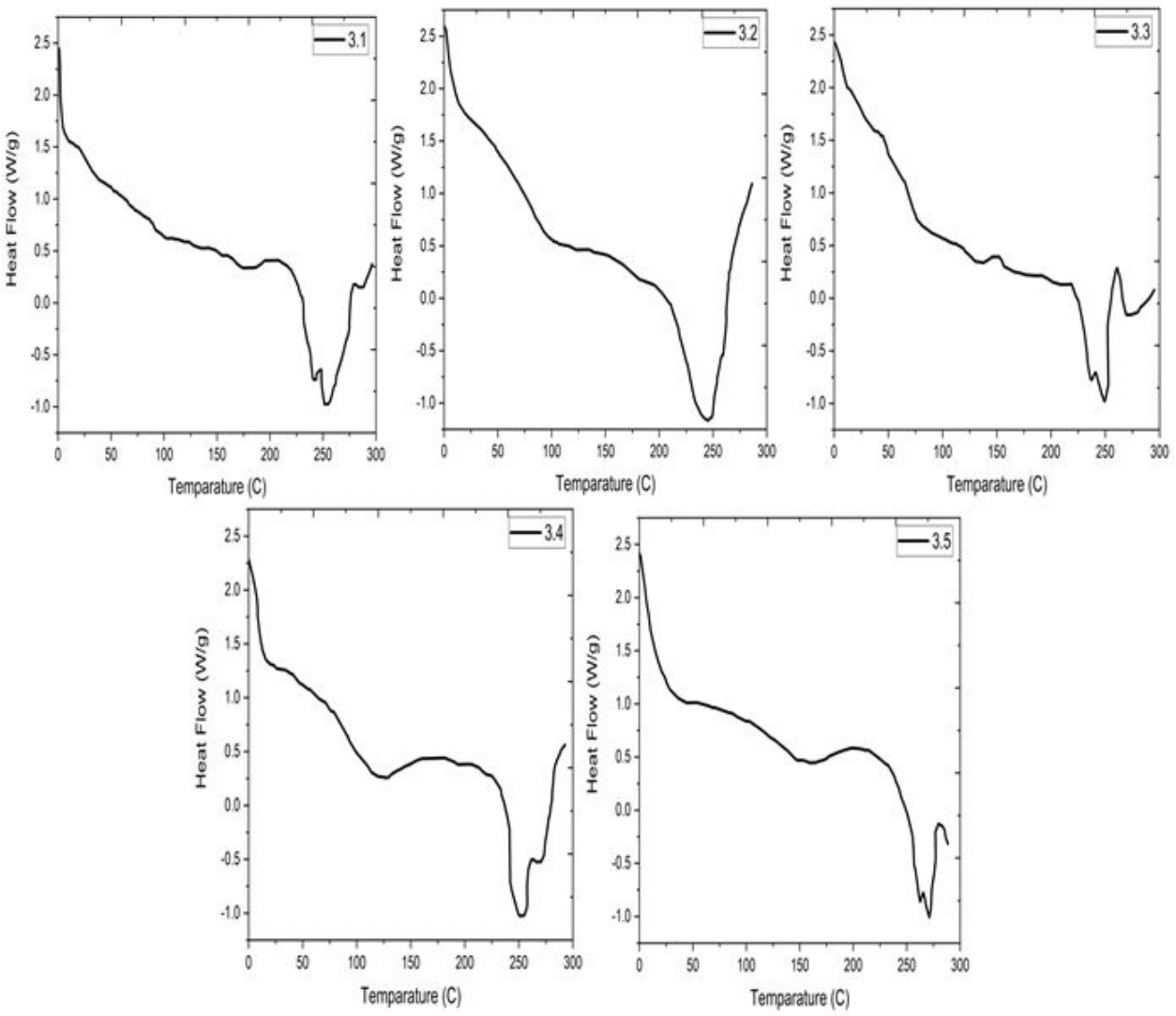

Fig.-3: DSC of Cross-linked Tri-polymers

SEM micrographs of structured cross-linked polymers are investigated and furnished in Fig.-4. The differences are obvious. The super absorbent polymer prepared from allyl tris buffer, acrylic acid and DVB cross-linking (Fig.-4b and c) have a solid amorphous nonporous shell. It can be observed that the fractographs are of a layer-like rough appearance (Fig.-4c), which indicates an adhesive interface. While the further two images of polymer thickeners prepared from DVB, acrylic acids and tris buffer monomers ATB shows different porosity. The highest porosity could be predicted from the monograph of polymer thickening agent cross-linked with ATB, the pores are associated with each other. The porosity can obviously be seen in the inflated view. However, in Fig.-4a aggregation of the porous structure can be seen and seems to be phase-separated.

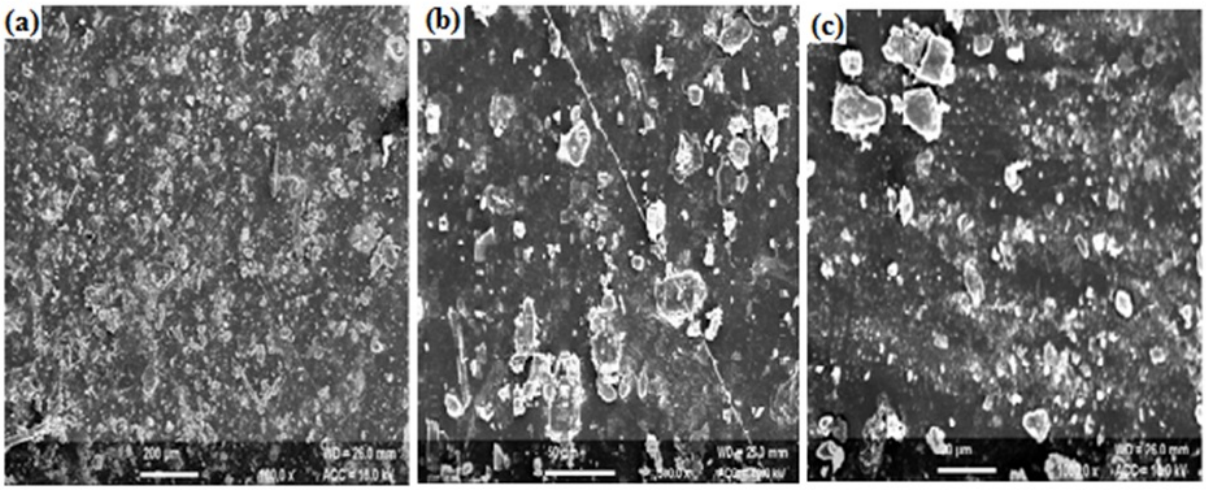

Fig.-4: SEM Micrographs of prepared SAP's shows for the Superabsorbent Polymer Cross-linked with PAA-ATB and Divinyl Benzene with Different Micro Sizes

Weight 0.5 g samples of absorbent polymer and placed into a beaker. The beaker is pre-weighed (W_0). The polymers are dipped in an excess quantity of water for the night to achieve the steadiness of swelling. After swelling, removes the excess volume of water waiting no fluid was drop-offs. The absorbed polymers are weighed (Ws) and the capacity of swelling is calculated by the equation:

$$\text{Swelling capacity} = (\text{swollen/dry}) \times 100\%$$
$$= (W_s / W_0) \times 100\%$$

Table-2: Absorbance Capacity of the Polymer

Weight of Cross-linking Agent (g)	Weight of Sample (g)	Weight of Swelled Polymer (g)	Swelling Capacity %
1.0	0.5	5.782	1057
0.8	0.5	8.012	1502
0.6	0.5	10.108	1922
0.4	0.5	11.614	2223
0.2	0.5	7.750	1450

CONCLUSION

Allyl tris buffer is hydrophilic, acrylic acid is also hydrophilic but DVB is hydrophobic. Since divinyl benzene is far in excess compared to allyl tris buffer, the polymer segments of acrylic acid/DVB form of one phase and ally tris buffer with acrylic acid form the immiscible second phase. By using the presented synthetic method clear rubbery gel polymer product is achieved. The surface morphology and thermal behavior are studied using SEM and TGA techniques, respectively.

The DSC of cross-linked SAP with allyl tris buffer shows a weight loss at 40°C. The super absorbent polymer synthesized from acrylic acid and the cross-linking agent has a solid smooth non-porous surface. Polymer started to degrade at about 150°C and completed nearly 260°C. The cross-linking was also supported by DSC analysis. According to swelling property of SAPs which are absorbs a larger amount of water. SAPs have many applications. Superabsorbent polymers have the capacity to intellect environmental variations, changes of pH, temperature. Hydrophilic networks are reactive to some molecules that can be used as in drug systems, hygienic products like diapers, and in controlled release drugs.[12] Superabsorbent polymers were also employed in various applications, such as absorbent paper products, bandages and surgical pads, wound dressings, and as chemical absorbents. Additionally, they are applicable to food packaging.[1]

The polymers are characterized using SEM patterns and FTIR spectra. The following conclusions are made based on the results and discussion.

i. FTIR spectra exhibit the cross-linking of monomers and cross-linker.
ii. From the swelling analysis, the newly synthesized polymers are absorbing more amount of water content, and they can be, therefore, employed as a super absorbent in various applications.
iii. In the DSC thermograms, weight loss may occur due to the degradation of the cross-linker.
iv. It can be observed that the SEM fractographs are of a layer-like rough appearance solid amorphous non-porous shell.

ACKNOWLEDGMENT

The authors are thankful to the Research Laboratory of Pramukh Swami Science and H D Patel Arts College, Kadi for providing research facilities.

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[RJC-5526/2019]

RJC

RASĀYAN *J. Chem.*
Vol. 13 | No. 1 |333 - 338| January - March | 2020
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

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For this research work allyl tris buffer, di-vinyl benzene, acrylic acid, ethanol and potassium thiosulphate all these reagents are of laboratory reagent grade. Basic glassware such as three-neck round bottom flask and mechanical stirrer and FT-IR spectrophotometer (Bruker alpha) were used.
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Table-1: Description of the Amount of Reagents

Experiment	3.1	3.2	3.3	3.4	3.5
Chemicals	Amount	Amount	Amount	Amount	Amount
Allyl tris buffer	1 g	0.8 g	0.6 g	0.4 g	0.2 g
Di-vinyl benzene	39 g	39.1 g	39.2 g	39.3 g	39.4 g
Acrylic acid	60 g	60.1 g	60.2 g	60.3 g	60.4 g
Ini.– $K_2S_2O_8$	1 g	1 g	1 g	1 g	1 g
Sol.-H_2O+C_2H_5OH	490 + 10 ml	490 + 10 ml	490 + 10 ml	490 + 10 ml	490 + 10 ml
Neut. - NaOH	7 pH	7 pH	7 pH	7 pH	7 pH

RESULTS AND DISCUSSION

The polymer thickeners are identified by Fourier Transform Infrared (BROOKER FT-IR) Spectrophotometer using their KBr pellets and the representative IR spectrum of the polymer 3.1 is presented in Fig.-1. The spectra show that the main characteristic peaks are at 2924 $cm^{-1}$and 2855 cm^{-1}. These are exhibited for the asymmetric stretching vibration of (-C=C-H Stretching) and asymmetric stretching vibration of N-H of the secondary amine group. The strong peak at 1696 cm^{-1} confirmed –C=O stretching of intermolecular hydrogen bonding of acrylic acid. Furthermore, the IR band at 1652 (merged, weak) cm^{-1} is owing to the characteristic stretching vibration of -C=C bond. The peak at 1396 cm^{-1} is accredited to O-H bending vibration. The band at 1156 cm^{-1} is recognized for –C - O stretching of ether bending vibration.
The thermo gravimetric analysis (TGA) of the polymers was run using TG (Perkin Elmer TGA 8000 Thermo Gravimetric Analyzer). The thermograms indicate that degradation of a polymer starts around 150°C and degradation completed above 200°C temperature. Thus, it can be concluded that the prepared polymer thickeners are thermally stable up to 150°C and few are up to 250°C. The canyons (converse peaks) are due to the fact that polymer has started to degrade as the impurity could be adhered in the form of aqueous phase or hydrocarbons and heat is liberated during degradation.
The cross-linking is also characterized by DSC analysis, as shown in Fig.-3. DSC of cross-linked thickener polymer indicates that a weight loss occurs within two stages.

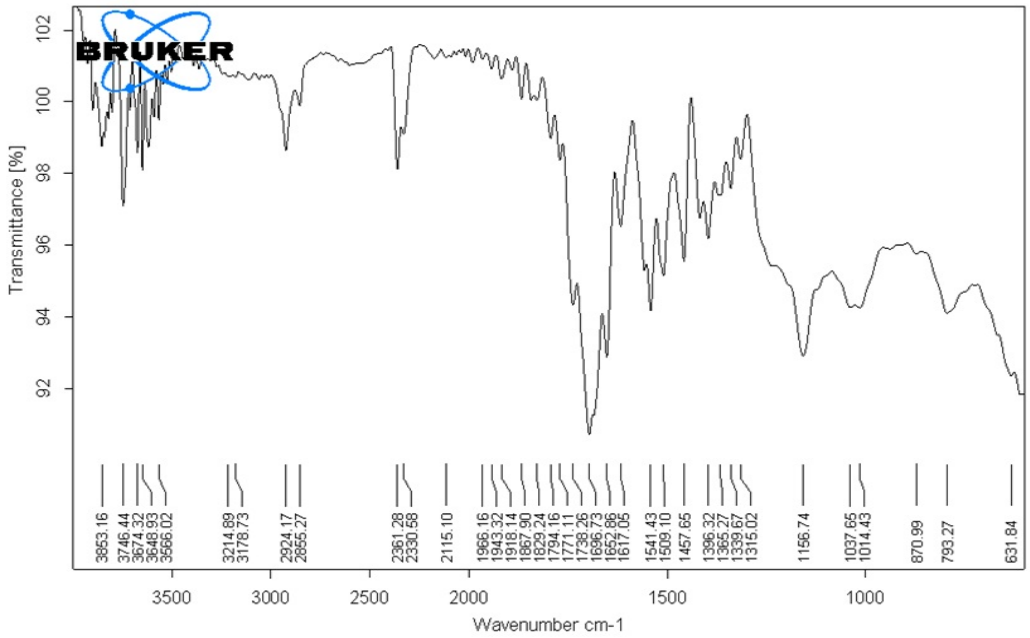

Fig.-1: FT-IR of Cross-linked Tri-polymer

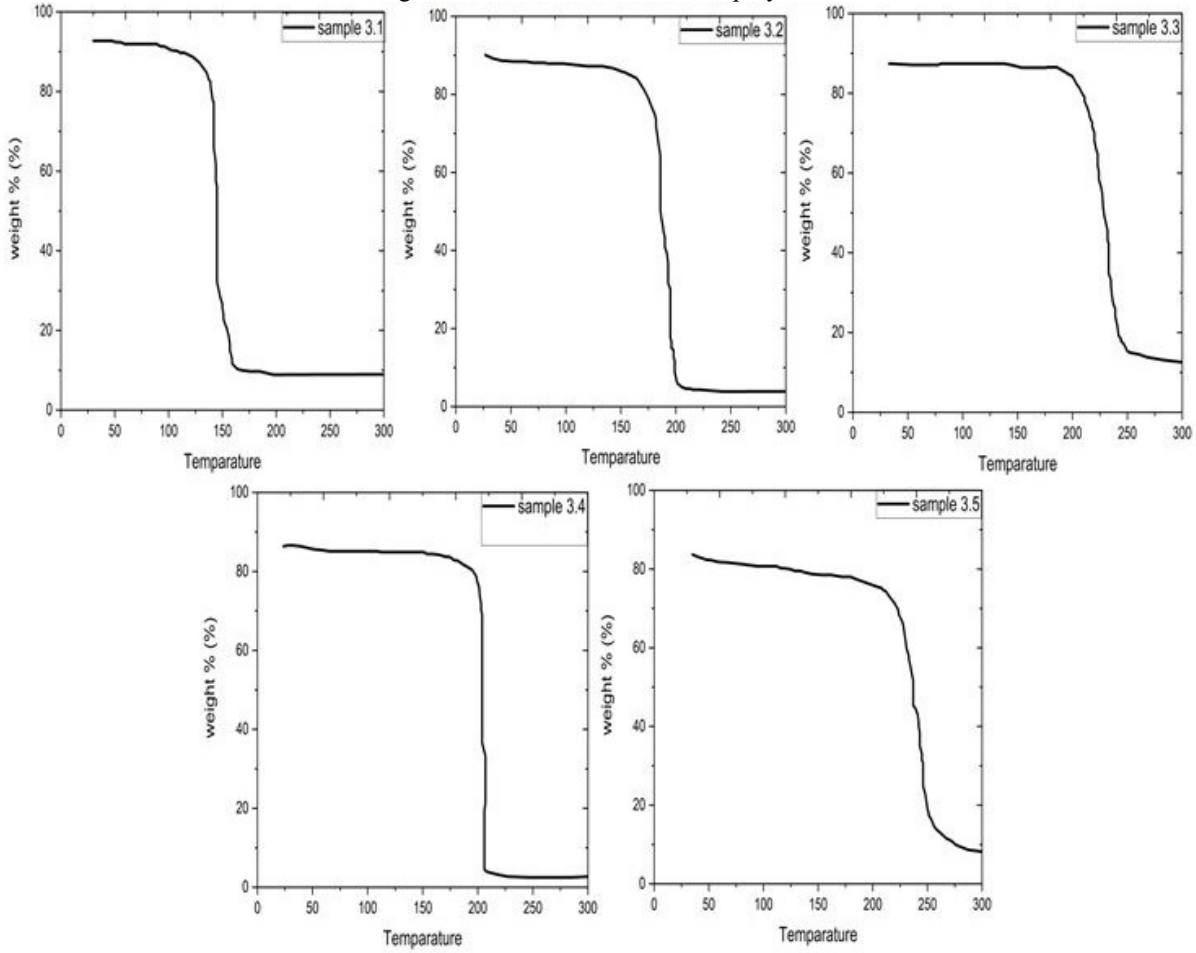

Fig.-2: TGA of Cross-linked Tri-polymers

Essentially, acrylic acid has the glass-transition temperature *(Tg)* is nearly 106°C, but due to cross-linked with DVB, there may be slightly changed in T_g. DSC thermograms designate that T_g of the polymer increases by about 115°C and is independent of the amount of allyl tris buffer. There is the hump in the bend and fix the midpoint in each curve as T_g. The initial stage shows weight loss is between 40°C and 125°C which might be due to the loss of absorbed and bound water. The next stage of mass loss started at 225°C and continued to 275°C, during which weight loss may be due to the degradation of the cross-linker.

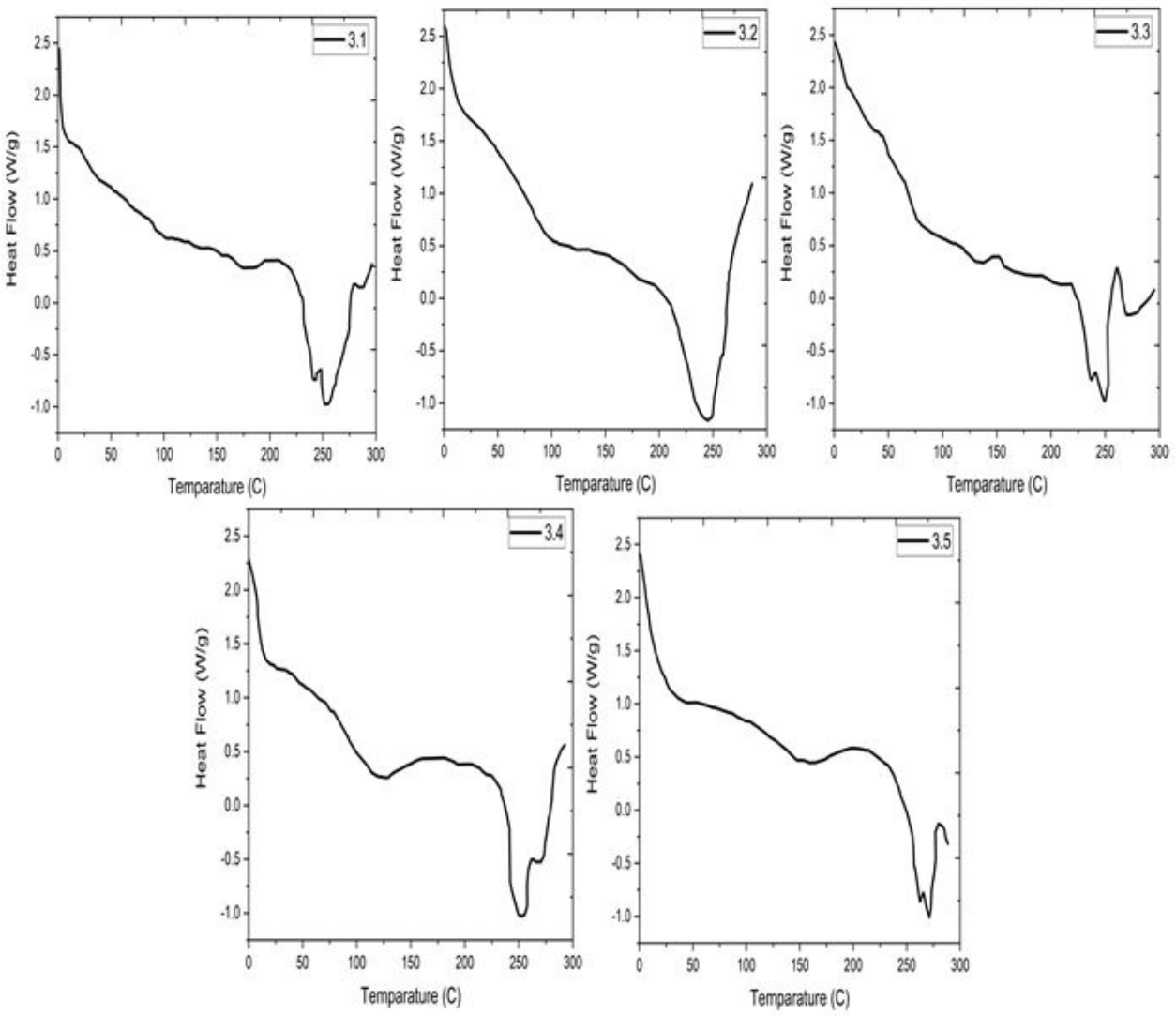

Fig.-3: DSC of Cross-linked Tri-polymers

SEM micrographs of structured cross-linked polymers are investigated and furnished in Fig.-4. The differences are obvious. The super absorbent polymer prepared from allyl tris buffer, acrylic acid and DVB cross-linking (Fig.-4b and c) have a solid amorphous nonporous shell. It can be observed that the fractographs are of a layer-like rough appearance (Fig.-4c), which indicates an adhesive interface. While the further two images of polymer thickeners prepared from DVB, acrylic acids and tris buffer monomers ATB shows different porosity. The highest porosity could be predicted from the monograph of polymer thickening agent cross-linked with ATB, the pores are associated with each other. The porosity can obviously be seen in the inflated view. However, in Fig.-4a aggregation of the porous structure can be seen and seems to be phase-separated.

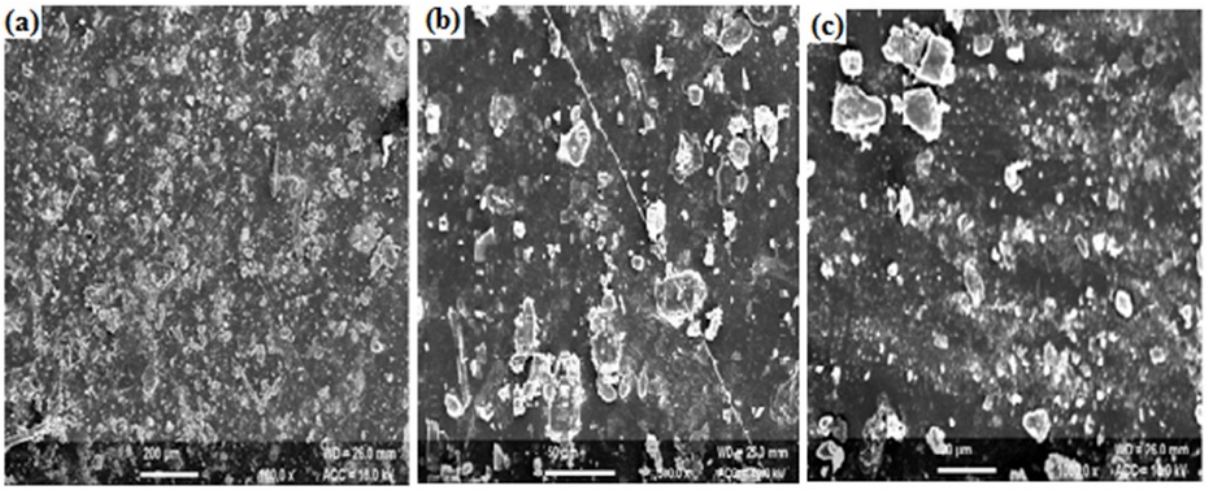

Fig.-4: SEM Micrographs of prepared SAP's shows for the Superabsorbent Polymer Cross-linked with PAA-ATB and Divinyl Benzene with Different Micro Sizes

Weight 0.5 g samples of absorbent polymer and placed into a beaker. The beaker is pre-weighed (W_0). The polymers are dipped in an excess quantity of water for the night to achieve the steadiness of swelling. After swelling, removes the excess volume of water waiting no fluid was drop-offs. The absorbed polymers are weighed (Ws) and the capacity of swelling is calculated by the equation:

$$\text{Swelling capacity} = (\text{swollen/dry}) \times 100\%$$
$$= (W_s / W_0) \times 100\%$$

Table-2: Absorbance Capacity of the Polymer

Weight of Cross-linking Agent (g)	Weight of Sample (g)	Weight of Swelled Polymer (g)	Swelling Capacity %
1.0	0.5	5.782	1057
0.8	0.5	8.012	1502
0.6	0.5	10.108	1922
0.4	0.5	11.614	2223
0.2	0.5	7.750	1450

CONCLUSION

Allyl tris buffer is hydrophilic, acrylic acid is also hydrophilic but DVB is hydrophobic. Since divinyl benzene is far in excess compared to allyl tris buffer, the polymer segments of acrylic acid/DVB form of one phase and ally tris buffer with acrylic acid form the immiscible second phase. By using the presented synthetic method clear rubbery gel polymer product is achieved. The surface morphology and thermal behavior are studied using SEM and TGA techniques, respectively.

The DSC of cross-linked SAP with allyl tris buffer shows a weight loss at 40°C. The super absorbent polymer synthesized from acrylic acid and the cross-linking agent has a solid smooth non-porous surface. Polymer started to degrade at about 150°C and completed nearly 260°C. The cross-linking was also supported by DSC analysis. According to swelling property of SAPs which are absorbs a larger amount of water. SAPs have many applications. Superabsorbent polymers have the capacity to intellect environmental variations, changes of pH, temperature. Hydrophilic networks are reactive to some molecules that can be used as in drug systems, hygienic products like diapers, and in controlled release drugs.[12] Superabsorbent polymers were also employed in various applications, such as absorbent paper products, bandages and surgical pads, wound dressings, and as chemical absorbents. Additionally, they are applicable to food packaging.[1]

The polymers are characterized using SEM patterns and FTIR spectra. The following conclusions are made based on the results and discussion.

i. FTIR spectra exhibit the cross-linking of monomers and cross-linker.
ii. From the swelling analysis, the newly synthesized polymers are absorbing more amount of water content, and they can be, therefore, employed as a super absorbent in various applications.
iii. In the DSC thermograms, weight loss may occur due to the degradation of the cross-linker.
iv. It can be observed that the SEM fractographs are of a layer-like rough appearance solid amorphous non-porous shell.

ACKNOWLEDGMENT

The authors are thankful to the Research Laboratory of Pramukh Swami Science and H D Patel Arts College, Kadi for providing research facilities.

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[RJC-5526/2019]

Callus Induction and Establishment of Cell Suspension Culture of Cumin (Cuminum cyminum L.)

Article *in* Journal of Plant Sciences · February 2020

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Research Article
Callus Induction and Establishment of Cell Suspension Culture of Cumin (*Cuminum cyminum* L.)

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Abstract

Background and Objective: Resistance to diseases is an important step for the establishment of a genetic transformation system in plants. In current research, callus induction and establishment of cell suspension culture were investigated in *Cuminum cyminum*. **Materials and Methods:** Callus inducted from hypocotyls segments. The experiment was arranged in a factorial experiment with 2,4-Dichlorophenoxyacetic acid (2,4-D) and Kinetin (KIN) different concentrations (0, 0.1, 1 and 10 µM) on MS medium. Callus was inducted 95.6% on MS medium supplements with 2, 4-D 0.1 and KIN 10 µM L^{-1}. The cell suspension culture was established from the callus of Cumin GC-4. Using different concentrations and combinations of 2,4-D and kinetin the growth patterns of cell suspension cultures were examined during a range of culture periods (0, 7, 14, 21 and 28 days). **Results:** The growth rates of cells were initially slow, but as the culture proceeded, they increased significantly and accumulated great amounts of biomass over a period of 28 days. **Conclusion:** Friable callus production is important in establishment of good quality of cell suspension. Researcher can take benefit from its investigation to manipulation genetically.

Key words: Cumin, callus culture, suspension culture, hypocotyls, genetic transformation, accumulation, biomass

Citation: S. Suthar Ramchandra, P.N. Bhatt and D.P. Bhatt, 2020. Callus induction and establishment of cell suspension culture of cumin (*Cuminum cyminum* L.). J. Plant Sci., 15: 54-63.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cumin (*Cuminum cyminum*) is a member of Apiaceae family. Cumin is originally cultivated in Iran, India, Pakistan, Egypt, Turkey and the Mediterranean region. India is a large producer and an earlier growing season than Syria, but 90% of national production is consumed internally. Callus tissue is an essential material in plant cell culture systems. When it is introduced into a liquid medium and agitated, the cells disperse throughout the liquid to form a cell suspension culture. Such cells are, in theory, totipotent and should also have a potential to synthesize any of the compounds normally associated with the intact plant[1]. As new cells are formed they are dispersed into the liquid medium and become clusters and aggregates. Cells in suspension can exhibit much higher rates of cell division than do cells in callus culture. Thus, cell suspension offers advantages when rapid cell division or many cell generations are desired or when a more uniform treatment application is required[2].

The techniques of plant cell culture facilitate the rapid production of variant cell lines. These variant cell lines are useful for research into the genetics and biochemistry of plant cells and also in biotechnology for the production of new plant varieties and secondary metabolites. Rapidly growing, fine suspension cultures or friable calluses are generally the most suitable for selection purposes. Where it is possible to regenerate plants from variant cells, selection techniques have potential for the production of crop varieties with new characteristics. Cell suspensions have also proven to be excellent starting materials for the isolation of protoplasts to be used in a wide range of applications including cell fusion and genetic manipulation[3].

Callus and suspension culture were reported earlier investigators with use of different plant growth regulators. Callus induction and shoot regeneration were investigated onto B5 medium containing 1.0 mg L^{-1} BAP, 0.2 mg L^{-1} NAA and 0.4 mg L^{-1} IAA with best result in Cumin[4]. The BAP is strongly recommended to initiates callus production and indirect regeneration pathways in cumin[5]. Organogenesis of cumin were reported in MS media with supplement TDZ plant hormone concentrations 0.5 and 0.1 and 0.5 mg L^{-1} 2,4-D[6,7].

Friable callus were obtained difficult in Cumin and further use in cell suspension. Presented study focus very well line of attack to achieve friable callus in good amount with a reduction of time. It used in protoplast isolation and fusion to acquire hybrid plants and genetically modifications and Establishment of Resistance variety.

MATERIALS AND METHODS

Study area: Seeds of GC-4 (Gujarat Cumin-4) were collected from Center for Research on Seed Spices. S.D. Agricultural University, Jagudan Gujarat, India. Research was carried out at plant tissue culture lab, established under potential for excellence, UGC grant. Research was conceded to solve wilt problem in Cumin and done in month of January, 2015 and 2016.

Surface sterilization of seeds: Seeds were first pre treated with 1% aqueous bavistin solution (Fungicide) for 15 min. Then they were soaked for 5 min in 20% solution of commercial bleach. Sterilization was done by using 0.1% $HgCl_2$ solution for 3 min and finally washed 4-6 times with auto-claved distilled water. Sterilized seeds were placed in sterile Petri dishes lined with moist filter papers and allowed to germinate. Individual Petri dishes were wrapped with parafilm to maintain it free from contamination and incubated under dark at 26°C. Almost 5-7 days after emergence, 4-6 mm long hypocotyls and epicotyls segments were cut from the seedling. These were incubated for the generation of callus.

Media optimization: A factorial experiment using total of 16 combinations for induction of callus from hypocotyls explants were done. The Callus Induction Medium (CIM) consisted of 2,4-Dichlorophenoxyacetic acid (2,4-D) and Kinetin (KIN) plant growth regulators at four concentrations ranging from 0, 0.1, 1 and 10 µM in the MS basal medium. The callus obtained from the hypocotyls was sub-cultured every 3 weeks in the respective medium for 4-6 cycles and maintained under fluorescent light (16 h/day).

Establishment of cell suspension culture: The establishments of Cumin cell suspension cultures were from three month old white/creamy friable callus as obtained. Callus obtained from hypocotyls of GC-4 on solid MS medium supplemented with 0.1 µM 2,4-D and 10 µM KIN were used for culture initiation. Approximately 1 g fresh weight of friable callus, which was still in its active growth phase was placed in 250 mL flasks containing 25 mL liquid MS medium[8] supplemented with 0.1 µM 2,4-D in combinations with 10 µM KIN. The flasks were placed on rotary shaker at 25°C under a 16 h photoperiod and agitated at 90 rpm. Sub-cultures were performed bi-weekly for the first 1 month and later at weekly intervals. For this the entire old medium was replaced by an equal volume of fresh medium by carefully decanting the top layer without agitating

and addition of fresh medium. Only small aggregates were decanted at every sub-culture. The ratio of cells to medium was 1:3 (v/v) in culture flasks.

Growth measurements

Viable cell count: Cell viability was determined by using trypan blue stain method. A small aliquot of cell suspension was mixed with 0.4% aqueous trypan blue and immediately observed under the microscope[9] viable cells extrude the dye and hence, are not blue. Dead cells absorb the dye and appear blue.

Cell number count: For measuring the increase in ratio of cells, cells were calculated (3×10^5 cells mL^{-1}) by using hemocytometer. To count the number of cells in Cell Suspension Culture (CSCs), these are treated with chromium trioxide. One volume of suspension cell culture were added to 4 volumes of 12% (w/v) aqueous chromium trioxide and heated at 70°C until the cells were stained and plasmolysed. The number of cells counted under microscope.

Packed Cell Volume (PCV): To calculate PCV, 10 mL of cell suspension culture were transferred in graduated conical centrifuge tubes then centrifugation at 2000xg for 5 min using a swing-out rotor. The cells were allowed to settle down (Pellet) and PCV was calculated. To study growth kinetics of cells in suspension cultures, a known number of cells need to be inoculated and this has to be uniform throughout the various replicates. The cell numbers can be established by calculating the ratio between number of cells and its packed cell volume. For this, a known volume of cell suspension was taken in replicates and transferred into graduated conical centrifuge tubes followed by centrifugation at 2000xg for 5 min using a swing-out rotor. The packed cell volume was determined as an average of several replicates and the number of cells counted using a haemocytometer. Thus, any volume used from this suspension could give total number of initial cells in the inoculums. For experimental purpose, different cell volumes (1, 2, 4 and 8 mL of 2 month old cell suspension) were taken and centrifuged as above. The cells that settled down (pellet) in each case were cultured in 10 mL of liquid MS medium supplemented with 0.1 μM 2,4-D, 10 μM KIN, 30 g L^{-1} sucrose, with pH 5.8 in a 100 mL flask on a rotary shaker at 110 rpm in fluorescent light (16 h/day) at 25-27°C. There were three replicates of 1, 2, 4 and 8 mL aliquots of cell suspension tested for 28 days. From these cultures, the PCV, fresh weights of the cells and cell number were determined at 0, 7, 14, 21 and 28 days of culture incubation.

Fresh weight: The cells that settled down (pellet) were cultured in 10 mL of liquid MS medium supplemented with 0.1 μM 2,4-D, 10 μM KIN, 30 g L^{-1} sucrose, with pH 5.8 in a 100 mL flask on a rotary shaker at 110 rpm in fluorescent light (16 h/day) at 25-27°C. From these cultures, the PCV and fresh weights of the cells were determined at 0, 7, 14, 21 and 28 days of cultures. Fresh weights were determined by collecting cells from pellet. Pellet was filtered and washed with water to remove the medium, drained under vacuum and weighted. There were three replicates of each treatment tested.

Statistical analysis: Experiments were repeated 3 times by using a complete randomized block design. Analysis of variance was carried out and differences between the means of the treatments were determined by Duncan's Multiple Range test at p-value of 0.05 and 0.01. The analysis of variance and means were carried out with the online statistical program, using Proc ANOVA and Proc Mean procedures. Doubling time and growth rates of CSCs were calculated and plot exponential regression graph by Doubling-Time 1.0 software. The population standard deviation was measured the variability of data in a population.

RESULTS AND DISCUSSION

Seed germination: When the seeds of Cumin GC-4 were placed for germination on moist filter paper only, they germinated within 4-7 days under dark condition. This was based on an earlier study[10]. Prior to this, other investigators had reported that seed germination of cumin required MS media with addition of gibberellic acid and increased concentration upto 2 mg L^{-1} for better result. Seed gemination were observed upto 75% and break seed dormancy[11]. In the present study, as high as 92% germination was achieved without having to grow the seeds on MS media with GA3, so MS media was omitted in this study (Fig. 1, 2).

Callus culture: In the presence of kinetin with 2,4-D and BAP with NAA in the callus induction medium, higher percentage of the explants produced callus. The hypocotyls segments

Fig. 1: Germinated Cumin seeds (between 4-7days)

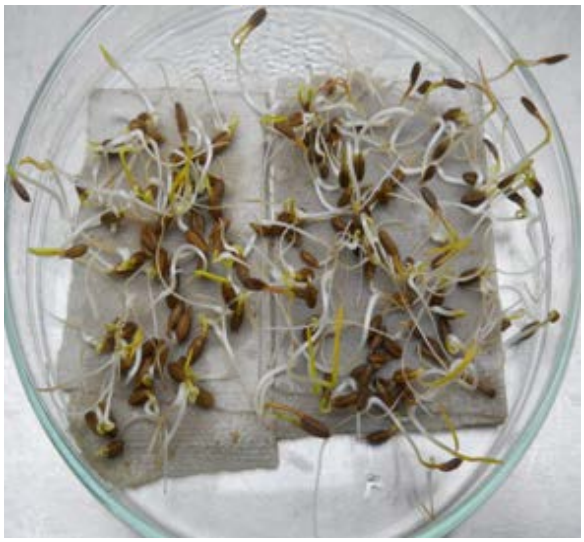


Fig. 2: Germinated Cumin seeds on filter paper after 7 days

(Fig. 3, 4), in contrast to the epicotyls explants were more responsive to the tested combinations of 2,4-D and kinetin (Fig. 5). As observed in case for callus induction and maintenance of other Apiaceae such as; fennel (*Foeniculum vulgare* Mill.), Cumin callus were more proliferative with kinetin and 2,4-D containing medium. Hypocotyls formed a great mass of callus during 3-6 weeks of the incubation on the Callus Induction Medium, CIM-14 having 0.1 µM 2, 4-D and 10 µM kinetin (Fig. 6, 7 and Table 1). On the other hand, only limited marginal callus was initiated from the epicotyls explants.

The callus obtained from the epicotyls explants, however, was comparable to the hypocotyls derived callus in the proliferation upon a sub-culture for an additional passage on fresh induction/maintenance medium. The callus from both explants was creamy to greenish creamy and soft/friable with smooth surface (Fig. 6). Similar to the present research also noticed that hypocotyls responded better than other Cumin seedling explants for callus production and proliferation with 95%. Earlier reporters were stated that 0.1 µM KIN and10 µM

Table 1: Callus initiation in Cumin from hypocotyls and epicotyls

Plant growth regulators (µM)		Callus initiation frequency (%)	
2,4-D	KIN	Hypocotyls	Epicotyls
0.0	0.0	0	0
0.1	0.0	20	18
1.0	0.0	43	40
10.0	0.0	62	56
0.0	0.1	31	34
0.1	0.1	49	55
1.0	0.1	59	56
10.0	0.1	62	58
0.0	1.0	41	43
0.1	1.0	61	58
1.0	1.0	62	62
10.0	1.0	71	68
0.0	10.0	79	76
0.1	10.0	95*	90
1.0	10.0	89	89
10.0	10.0	90	93

Table 2: Analysis of variance for callus initiation from hypocotyls percentage in Cumin (*Cuminum cyminum* L.)

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares
Replications	2	1.167	0.583
Treatments	16	30360.146	2024.010
Error	30	20.167	0.672
Total	48	-	-

Table 3: Comparison of treatment means with critical difference (0.05)

Treatment numbers	T 14	T 16	T 15	T 13	T 12	T 4	T 11	T 8	T 10
Treatment average	95	90	89	79	71	62	62	62	61
Critical Difference (CD) compared	a	b	b	c	d	e	e	e	e

a: 100-95, b: 94-89, c: 88-79, d: 78-71, e: 72-65

Fig. 3: Hypocotyls segments of Cumin

Fig. 5: Callus induced on CIM-14 with 0.1 µM 2,4-D and 10 µM Kinetin (after 14 days)

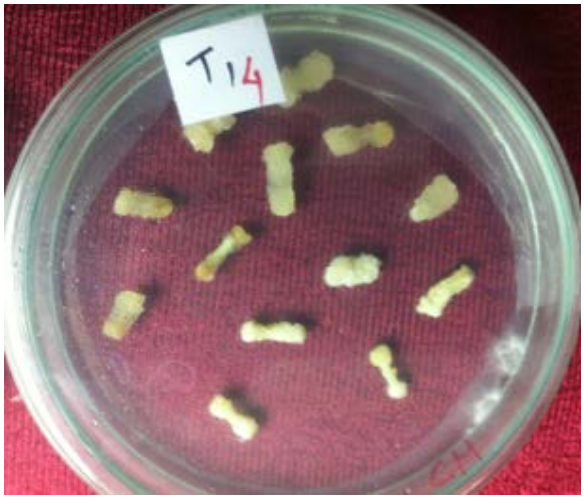


Fig. 4: Callusing explants on CIM after 7 days of inoculation

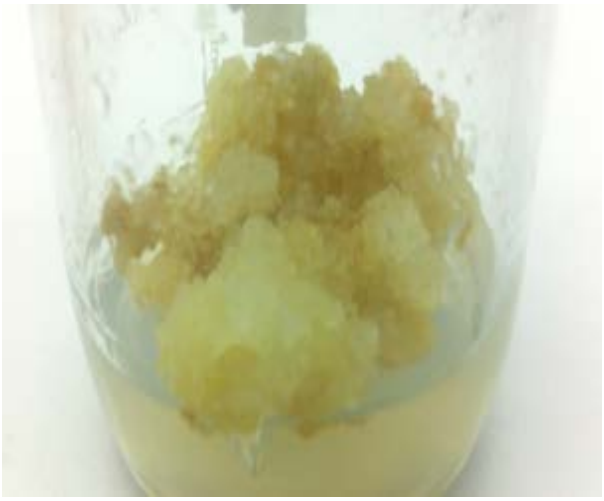


Fig. 6: Sub-culture of callus on CIM-14 with 0.1 µM 2,4-D and 10 µM Kinetin (after 28 days)

2,4-D growth regulators initiated cell suspension in Cumin in liquid medium[12]. Most of investigators used 4 µM 2,4-D alone or plus 2 or 4 µM KIN on Cumin studies[13-16].

Analysis of variance in a total 16 treatments was observed with different concentrations of 2,4-D and KIN (Table 2). Coefficient of variation is 1.423. Treatments were found to be significant at 1 and 5% level of significance Critical Difference (CD) (0.01) = 1.841 CD (0.05) = 1.367. Treatment-14 (CIM-14) was selected for further experiments (Table 3).

Treatment average: Analysis of variance in a total of 16 treatments was observed with different concentrations of 2, 4-D and KIN for epicotyls. Most significant were a, b, c, d and e shown in Table 4. Treatment average 95 in treatment No. 14 was best combination of hormones to produced superior amount of callus in Cumin. Coefficient of variation was 1.105.

Treatment was found significant at 1 and 5% level. CD (0.01) = 1.383 and CD (0.05) = 1.027.

Sub-culture: The CIM-14 (Callus Induction Medium) gave very profuse callus proliferation compared to other combinations. The callus was creamy to greenish white in color and very friable, which is most appropriate for initiation of cell suspension cultures (Table 5). As a result, CIM-14 was

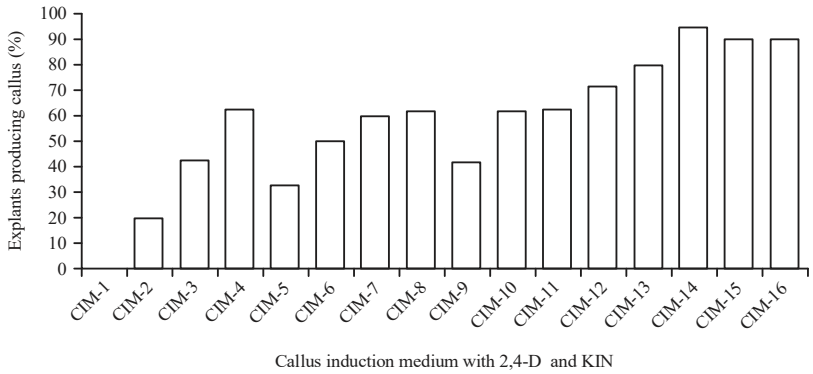


Fig. 7: Callus induction from hypocotyls

Table 4: Analysis of variance for callus initiation from epicotyls percentage in Cumin (*Cuminum cyminum* L.)

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares
Replications	2	2.625	1.313
Treatments	16	28917.000	1927.800
Error	30	11.375	0.379
Total	48	-	-

Table 5: Characterization of Cumin callus induced from hypocotyls

Callus induction medium	2,4-D (μM)	KIN (μM)	Characterization
CIM-1	0.0	0.0	---
CIM-2	0.1	0.0	Friable creamy
CIM-3	1.0	0.0	Friable creamy
CIM-4	10.0	0.0	Friable creamy
CIM-5	0.0	0.1	Compact greenish
CIM-6	0.1	0.1	Friable creamy
CIM-7	1.0	0.1	Compact greenish
CIM-8	10.0	0.1	Friable creamy
CIM-9	0.0	1.0	Compact greenish
CIM-10	0.1	1.0	Friable greenish
CIM-11	1.0	1.0	Friable creamy
CIM-12	10.0	1.0	Friable brown
CIM-13	0.0	10.0	Compact greenish
*CIM-14	0.1	10.0	*Friable greenish
CIM-15	1.0	10.0	Friable greenish
CIM-16	10.0	10.0	Friable brown

CIM: Callus induction medium, *Selected CIM-14 for subsequent experiments

Table 6: Growth kinetics of Cumin cell suspension cultures

	Aliquot (1 mL)		Aliquot (2 mL)		Aliquot (4 mL)		Aliquot (8 mL)	
Days	PCV	Fresh weight (mg)	PCV	Fresh weight (mg)	PCV	Fresh weight (mg)	PCV	Fresh weight (mg)
0	0.41	4.0	0.83	9.0	0.96	10.2	1.46	14.3
7	0.56	6.2	0.95	10.1	1.21	12.4	1.56	15.0
14	0.72	8.2	1.20	12.2	1.25	12.5	1.67	16.2
21	0.95	10.1	1.23	12.1	1.42	14.0	1.97	18.0
28	1.20	13.2	1.28	12.7	1.56	16.3	2.10	20.0
Total increase after 28 days	0.79	9.2	0.45	3.7	0.60	6.1	0.64	5.7
Initial cell number (cells mL^{-1})	3×10^5		6×10^5		1.1×10^6		2.2×10^6	
Growth rate	0.038		0.0154		0.0157		0.0139	
Doubling time (day)	0.75		1.86		1.66		2.22	

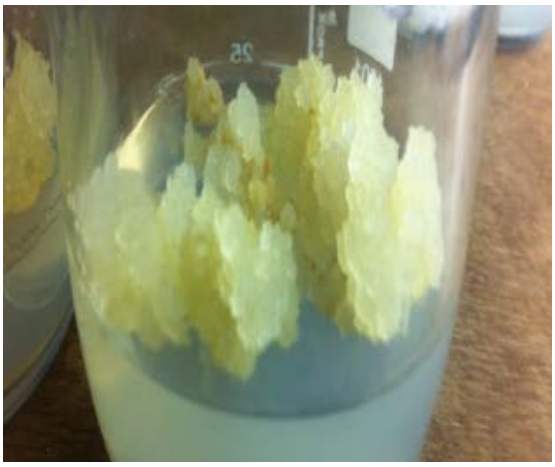


Fig. 8: Friable callus of cumin

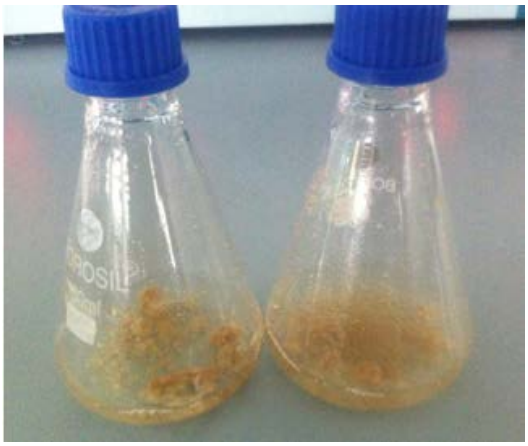


Fig. 9: Cell suspension culture in 0.1 µM 2, 4-D and 10 µM KIN medium

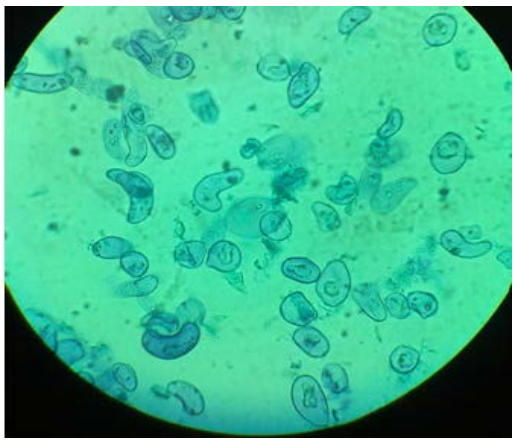


Fig. 10: Cumin cells suspension under inverted microscope stained for viability (Non-viable cells appear dark blue)

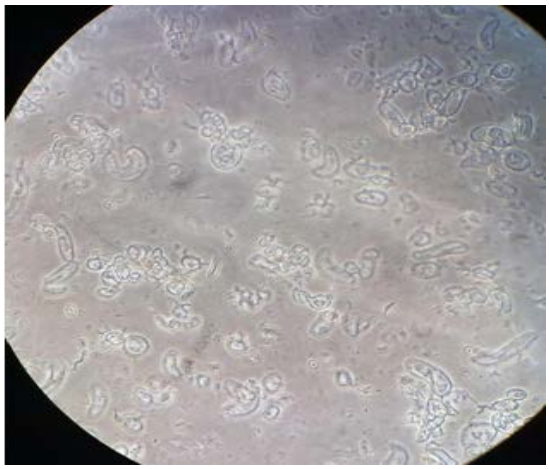


Fig. 11: Cumin cells suspension under inverted microscope stained for viability (viable cells remain unstained)

selected from all the 16 combinations tried for sub-culture and the callus were maintained on this medium for all subsequent experiments.

Cell suspension culture: The establishment of Cell Suspension Culture (CSC) was only possible by using friable callus. The CSC was initiated from single cells, callus clump and friable calli (Fig. 8, 9). At an early stage of CSC, large, elongated and highly vacuolated cells with thick cell wall were observed (Fig. 10, 11). During the subsequent frequent sub-cultures, they were stepwise eliminated. Extrusion of dye leaving cells unstained indicated viability, the ratio of which gradually increase to unviable blue cells in cell cultures over successive cycles. With repeated bi-weekly/weekly sub-cultures, it was possible to achieve a suspension consisting of small cell aggregates. The growth dynamics of CSCs was determined using small cells aliquots (1, 2, 4 and 8 mL) from 2 months old CSCs which were inoculated in fresh cell culture medium. It consisted of MS+0.1 µM 2,4-D and 10 µM KIN. Each cell suspension consisted of a total volume of 10 mL and aliquots with cell density of 3×10^5 cells mL^{-1} were added to the cell culture medium (Fig. 12). Four replicates were observed for the experiment.

To establish a well growing cell suspension culture it is necessary to inoculate at least 10^4 cells mL^{-1}, otherwise the cells may not divide. This value depends, however, also on the aggregate size. The aggregate size has an influence on natural product formation as well. The reason why plant cells need such relatively high cell densities for undergoing division surely is that they lose hormones and/or vitamins and nutrients to the surrounding medium.

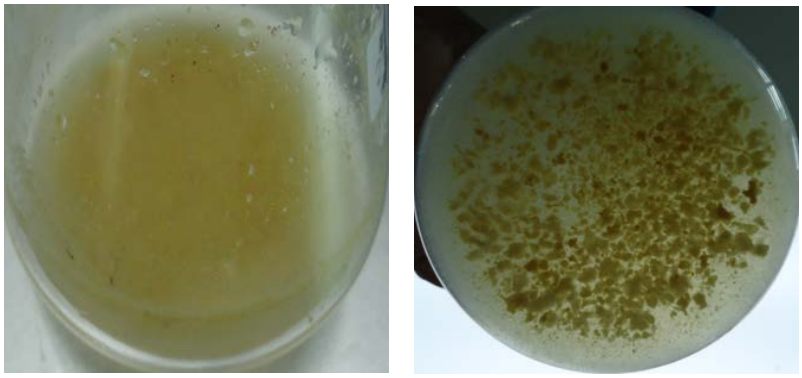
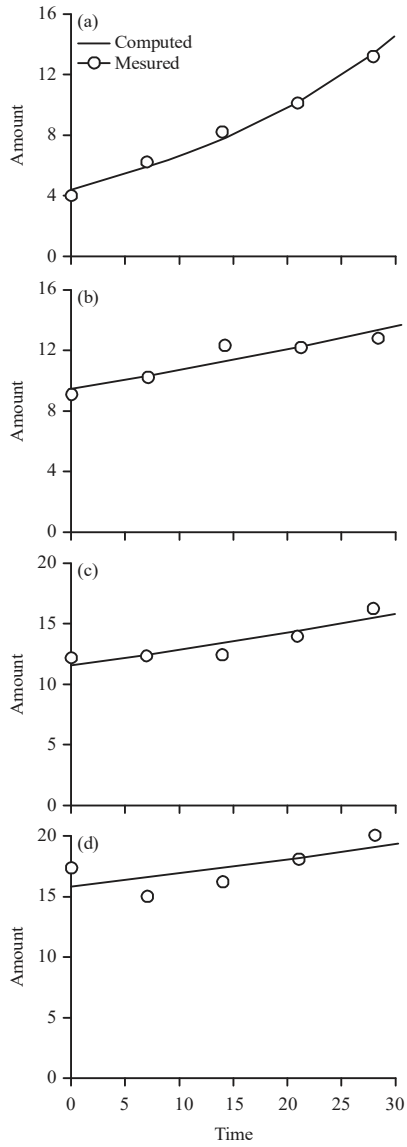


Fig. 12: Cell suspension culture in 0.1 µM 2,4-D, 10 µM KIN after 2 months



A cell density of 3×10^5 cells
Doubling time (Days) = 0.75 = ln (2)/0.0393
Growth rate = 0.0393
Growth rate = Number of doublings that occur per unit of time
Equation: Amount = $4.4715\times e^{0.0393*time}$
At t = 0, calculated cell concentration = 4.4715

B cell density of 6×10^5 cells
Doubling time (Days) = 1.86 = ln (2)/0.012
Growth rate = 0.0154
Growth rate = Number of doublings that occur per unit of time
Equation: Amount = $9.4316\times e^{0.012*time}$
At t = 0, calculated cell concentration = 9.4316

C cell density of 1.1×10^6 cells
Doubling time (Days) = 1.66 = ln (2)/0.0132
Growth rate = 0.0157
Growth rate = Number of doublings that occur per unit of time
Equation: Amount=$11.9646\times e^{0.0132*time}$
At t = 0, calculated cell concentration = 11.9646

D cell density of 2.2×10^6 cells
Doubling time (Days) = 2.22 = ln (2)/0.0068
Growth rate = 0.0139
Growth rate = Number of doublings that occur per unit of time
Equation: amount = $15.7344\times e^{0.0068*time}$
At t = 0, calculated cell concentration = 15.7344

Fig. 13(a-d): Detail of doubling time, growth rate and cell concentration calculated by software for different cell density of CSCs of Cumin

The growth dynamic of CSCs was determined at various cell densities on CIM-14 medium containing 0.1 μM 2,4-D and 10 μM KIN lacking agar. The data on PCV and fresh weight measured every week indicated that the cells grew and multiplied rapidly with a doubling time of 0.75 days. Elongated and divided cells were observed more in 21 days.

Fresh weight and PCV of the cells increased nearly 3 folds and a cell doubling time of 0.75 and growth rate of 0.038 with initial inoculum density of 3×10^5 cells mL^{-1} (Table 6) with a doubling time of 1.86 days was calculated with 2 folds increased fresh weight mean 11.16% from 6×10^5 cell mL^{-1}, growth rate 0.0154 was calculated during 28 days. With an increase in the initial inoculums density, the growth rate decreases and there is a corresponding increase in the doubling time to the extent that with 8 fold increase of initial inoculum, the growth rate fall three fold (0.0139) and the doubling time goes up three fold (2.22 days) as shown in Table 6 and Fig. 13a-d. Earlier, investigator reported a doubling time of 2.0-2.2 days for Cumin cell suspension in B5 medium[15] and also reported 2-2.5, 4.7 and 3-6 days doubling time respectively in wheat cell cultures[17-19]. In present study, the minimum doubling time was as short as 0.75 and maximum was 2.22 days as shown by Ahmed *et al.*[15]. These differences could be due to the difference in medium composition (here MS medium was used instead of B5) or the initial cell density of the suspension.

In this study, it described callus culture and the establishment of cell suspension cultures from white and friable callus, which was previously obtained from hypocotyls segments of cumin GC-4. Using different concentrations and combinations of the cytokinin KIN and auxin 2,4-D (0.0, 0.1. 1.0 and 10 μM) the growth patterns of the cultures were examined during a range of culture durations. The growth rates of cells were initially slow, but as the culture proceeded, they increased significantly and accumulated great amounts of biomass over a period of 28 days. Medium containing high kin (10 μM L^{-1}) and 2,4-D (0.1 μM L^{-1}) induced higher rates of cell division than the medium containing low KIN (0.1 μM L^{-1}) and 2,4-D (10 μM L^{-1}) or the control. After selection of small aggregates and single cell culture at 15 days intervals for 2 months, homogeneous and light yellow CSCs, composed of single and small cells aggregates were established. The optimized culture conditions obtained from the present study will then be used in resistance cell selection against pathogen toxin experiments. Investigated study promotes genetic manipulation.

CONCLUSION

The MS basal medium with 1.0 μM 2,4-D and 10 μM Kinetin was found most suitable for high callus induction from epicotyls and hypocotyls as explants of Cumin. Cell suspension cultures were derived from these calli by transfer to liquid MS medium with 1.0 μM 2,4-D and 10 μM Kinetin. The information is imbibed in this research article indicate the role of 2,4-D and kinetin in differentiations of cumin.

SIGNIFICANCE STATEMENT

Epicotyls and hypocotyls of Cumin were good sources of callus induction. Friable callus production is important to establishment of good quality of cell suspension. Researcher can take benefit from its investigation to manipulation genetically.

ACKNOWLEDGMENT

The authors wish to acknowledge the principal Dr. Ajay Gor and staff of the Department of Biotechnology, P.S. Science and H.D. Patel Arts College, Kadi, India, for granting us access to their equipments.

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INDIAN JOURNAL OF SCIENCE AND TECHNOLOGY

RESEARCH ARTICLE

Isolation, screening and identification of Lipase producing fungi from cotton seed soapstock

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OPEN ACCESS

Received: 09.07.2020

Accepted: 26.09.2020

Published: 09.10.2020

Editor: Dr. Natarajan Gajendran

Citation: Patel GB, Shah KR (2020) Isolation, screening and identification of Lipase producing fungi from cotton seed soapstock. Indian Journal of Science and Technology 13(36): 3762-3771. https://doi.org/10.17485/IJST/v13i36.1099

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Funding: None

Competing Interests: None

Published By Indian Society for Education and Environment (iSee)

ISSN
Print: 0974-6846
Electronic: 0974-5645

Abstract

Background/Objectives: The present study was focused to exploit the indigenous strains of fungi isolated from cotton seed soapstock for the production of the extracellular lipase through submerged fermentation technique. **Methods/ Statistical analysis:** Cotton seed soapstock samples used in the study contains gelatinous oil richer chemical constituents. In addition, their enrichment and diluted materials were used for the isolation of lipase producing microorganisms on tributyrin agar plates. All isolates were lipase positive confirmed by a qualitative plate assay. Quantitative estimation of Lipase production activity was measured spectrophotometrically using p-nitrophenyl palmitate (p-NPP) as a substrate. In order to exploit the isolated fungal strain for industrial usage, other cellulase and protease enzymes were tested by plate assay. Morphological and molecular characteristics of selected isolates were studied. **Findings:** From enriched soapstock samples, a total of 49 cultures were isolated among them 19 fungal isolates were screened for lipase, cellulase, and protease activity qualitatively by plate assay. Out of 19, six fungi were selected based on their lipase activity. Highly potent *Fusarium solani* 7F had the ability to produce 5.95 U/mL/min. crude lipase whereas *Aspergillus niger* 13F has 4.2 U/mL/min. after 4days incubation at 30^{o}C. Potent fungi culture was identified by morphological, cultural, and molecular characteristics (18s rRNA gene sequence and phylogenetic analysis) revealed them as *Penicillium griseofulvum* 5F, *Aspergillus flavus* 6F, *Fusarium solani* 7F, *Aspergillus niger* 12F, *Aspergillus niger* 13F, and *Aspergillus terreus* 17F. **Novelty:** Fungi was the first time reported and isolated from cotton seed soapstock materials. In future studies, this enzyme will be used in the degradation of soapstock and also in the production of biosurfactant from soapstock.

Keywords: Cotton seed soapstock; Fusarium solani 7F; p-nitrophenyl palmitate; Tributyrin agar plates; degradation

1 Introduction

Lipases (triacylglycerol ester hydrolases E.C.3.1.1.3.) are one of the most important industrial enzymes catalyze both the hydrolysis and the synthesis of esters from

glycerol and long-chain fatty acids. These enzymes, under specific condition to catalyze reversible reactions: interesterification, aminolysis and transesterification reactions [1,2]. Novel enzymes research of lipase may provide greater understanding of previously discovered enzymes and their functional significance using molecular tools that may be used as parts of the microbial pool for production of lipase in research at laboratory and industries level [3]. Research aspects, reported lipase producing fungi were *Humicolal anuginosa* [4], *Fusarium* sp. [5–7], *Mucor* sp. [8], *Aspergillus* sp. [9,10] *Rhizopus oryzae* [6,11], *Colletotrichum gloeosporioides* [12], *Alternaria dianthicola*, *Curvularia* sp., *Penicillium* sp., *Trichoderma viridae*, *Macrophomina phaseolina* [6], *Hypocrea pseudokoningii* [13] etc. Lipases are a ubiquitous enzyme that is found widely in a variety of natural sources industrial wastes, vegetables oil processing factories, soil contamination with oil, etc [14]. Lipid is a large constituent of the earth's biomass and has its application in various industries like in detergents, dairy and textile, production of surfactants, oil processing and biodiesel of microbial origin [15,16].

Cotton is a source of fiber, oil and protein. Oil-rich seeds are the vigor of pathogenic fungi in the process of biodeterioration of seed may be related to their degree of lipase production [6]. Soapstock is a gelatinous dark brown undesirable chemical compound which is separated from the oil refinery [17]. Soapstock will account for higher values for 5 to 10% of the crude oil mass and high concentration of free fatty acids and these byproducts use for fungal lipase production in SSF *Aspergillus niger* [18]. Soapstock from alkali refining is a source of fatty acids, but it also presents a handling, storage, and disposal problem. It is generated at a rate of ~6% of the volume of crude soybean oil caustic refined [19].

The production process, oils from agro-industrial waste can come forward as efficient enzymes inductor reducing the production cost. Additionally, this process can reduce environmental problems related to agro-industrial disposal [20]. Screening for new microorganisms and their lipolytic enzyme will open simple routes for synthetic processes and consequently new and faster ways to solve environmental problems. In this regard, the biotechnological process that can added value to waste by biodiesel production via lipase enzyme esterification [21,22]. On other hands microbial treatment to soapstock, *Staphylococcus* sp. strain produced biosurfactants and extracellular lipase, when soapstock was used as an alternative carbon source. These potent metabolic product biosurfactant and lipolytic enzymes were potentially applicable in soap stock treatment [23]. *Pseudomonas aeruginosa* strain was utilized Soybean oil soapstock as the sole carbon source for the production of rhamnolipids [24]. *Candida antarctica* and *Candida apicola* were produced glycolipids when supplemented with soapstock [25]. *Oospora lactis* fungi utilized cotton oil soapstock as carbon source and produced lipase enzyme. During fungi cultivation on soapstock, enzymatically hydrolysis of fat was taken place on the approach of biological alteration of cotton oil soap stock [26]. Lipase producing fungal strains, *Aspergillus*, *Penicillium*, *Trichoderma* and *Mucor* were isolated from palm oil mill effluent composts [27]. Where Lipase producing *Bacillus licheniformis* and *Bacillus pumilus* were isolated from cotton soapstock which will used in enzymatic degradation of soapstock [28]. Lipase produced by *F. solani* isolated from leaves decomposed in an aquatic environment has great potential to application in biodiesel production by transesterifcation of vegetable oils, as well as food industries in the production of fatty acid esters by hydrolysis and esterifcation. In the present study, maximum lipase activity was achieved by *Fusarium solani* using cotton oil as substrate and there is no any work related to isolation of lipolytic fungi by using cotton soapstock as a natural source.

This study was conducted to isolate lipase producing fungi that were enriched and screened on tributyrin agar plates. Culture was further analyzed for cellulase and protease production by plate assay. The fungal strain was identified on the basis of morphological characteristics and molecular 18s RNA sequence (Genetic Characterization) of the cultures using manual partial gene sequencing. In future study lipase will used in degradation of soapstock and produce industrially important products.

2 Materials and Methods

2.1 Sample collection

Lipolytic fungal strains were isolated from cotton seed Soapstock samples collected from different cotton seed oil refinery industries situated at Kadi (North Gujarat), India. All the soapstock samples were collected at the site of Washer discharge end of the pipe in sterile containers.

2.2 Enrichment process of Soapstock sample

For enrichment B/H (Bushnell–Haas) medium was used [29–31]. 10 grams of Cotton seed oil soapstock samples were enriched in 100 ml of B/H mediums for the growth of microbes. Broths were incubated at 30^{o}C in static condition for 5 days. From each enriched samples, 1ml of samples were inoculated to 100 ml of the Trybutyrine broth medium. TBA broth was incubated at 30^{o}C, in static condition for 48 hours. Enrichment was performed over 7 days of incubation.

2.3 Isolation of Lipolytic Fungi

For isolation of fungi, A series of dilutions from 10^{-1} to 10^{-10} were prepared from enriched sample. These dilutions were used in spread plate method. The diluted samples (100 μl) were pipette and spread on Tributyrin agar plate and incubated at 30^{o}C for 7 days. Isolated fungal colonies were sub-cultured and purified on the TBA medium. Isolated cultures were preserved at 4^{o}C temperatures in Potato dextrose agar slants for further works.

2.4 Qualitative screening of Lipase producing fungi

For screening, Qualitative plate assay was performed according to the method reported earlier for lipase-producing strains selection [31]. Isolates plugs were inoculated on tributyrin agar plates and incubated at 30°C for 5 days. The clear zone was observed due to the hydrolysis of tributyrin by lipase.

2.5 Lipase enzyme assay

2.5.1 The composition of production medium for Lipase enzyme

In 250 ml Conical flask 100 ml of production medium [31] were prepared for fungal culture. Production medium for lipase contain 0.5% Peptone, 0.5% Yeast extract, 0.5% NaCl, 1% Cottonseed Oil, pH-7.

2.5.2 Quantitative analysis of Lipase enzyme under submerge fermentation

Fungal cultures that showed Positive lipase production in plate assay were subjected to quantitative analysis. 5 days old cultures grown on the TBA medium were used for inoculation. Two plugs of fungal culture were inoculated into 100 ml of production medium for lipase production. Cultures were incubated at 30°C and 100 rpm for 5 days. The enzyme assay was performed according to the method reported by Patel and Shah, 2018 [32]. The culture filtrate was removed from each flask at every 24 hrs interval and centrifuged at 10,000 rpm for 10 min at 40°C. Supernatant were used for enzyme assay. By using p-nitro phenyl palmitate (p-NPP) as a substrate, lipase production was determined by a spectrophotometric assay. Lipase hydrolyzed p-NPP to give p-NP which gave yellow color and absorbance of which was measured spectrophotometrically at 410 nm against enzyme free blank.

2.5.3 Assay of enzyme

The substrate solution preparation: solution A (40 mg of p-NPP dissolved in 12 ml isopropanol) and solution B (0.1 g of gum Acasia and 0.4 ml of triton X-100 dissolved in 90 ml of distilled water). The substrate solution was prepared by adding 1 ml of solution A to 19 ml of solution B drop wise with constant stirring to obtain an emulsion that remains stable for 2 h. The assay mixture contains 1 ml of the substrate, 0.5 ml of buffer (Potassium phosphate buffer, pH 7, 0.1 M) and 1 ml of the enzyme. The volume was made up to 4 ml with distilled water. Incubate at RT for 5 min. Lipase activity was stopped by adding 1 ml 0.2M NaOH. Lipase activity was measured by monitoring the hydrolysis of 5mM p-nitro phenyl palmitate (pNPP) buffered with 0.1 M Potassium Phosphate buffer (pH 7.0) at 410 nm for 5 min. One unit of lipase enzyme activity defined as 1mM of p-NP liberated per min under the assay conditions.

2.6 Enzyme profile

Qualitative screening for Cellulase and Protease were done in nutrient agar media containing 1% carboxy methyl cellulose and 1% skim milk substrates respectively. Culture plug was inoculated and incubated at 30°C for 5 days. Zone of clearance was observed around the colonies due to the utilization of the particular substrate.

2.6.1 For Cellulolytic activity

As per method performed earlier [3], culture were grown in medium containing 1% carboxy methyl cellulose. After incubation 0.1 % congo red staining solution was added in CMC plates, discard stain after 5 min and the plates were destained by 1M NaCl solution with continuous stirring for 15-20 min. The clear zone around colonies indicated cellulose hydrolysis.

2.6.2 For Proteolytic activity

1% skim milk substrate containing nutrient agar media were used for screening of protease [33]. Culture plugs was inoculated and incubated at 30°C for 5 days and observed for clear zone around colonies due to protein hydrolysis.

2.7 Identification of the fungal isolates: morphological and molecular characteristics

The potent isolates showing the maximum zone of clearance were selected for further analysis. On potato dextrose agar medium, morphological and cultural characteristics of the isolates were studied such as the color of hyphae, type of hyphae, shape and the characteristics of spore, using microscopic examination. Molecular characterization of potent fungal strains was done by 18s rRNA partial gene sequencing analysis. It was carried out at the Biogene department of Gujarat State Biotechnology Mission (GSBTM), Gandhinagar. Fungi culture isolates were identified through partial gene ITS region sequence and 18S rRNA sequence using ITS 1/ITS 4 primer. The 18S rRNA gene sequence was used to carry out BLAST with the nr database of NCBI gene bank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using the RDP database and the phylogenetic tree was constructed using MEGA X.

2.8 Statistical analysis

In all experiments, three sets of reading were taken. Data, where appropriate, were subjected to two- way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and student's test using Graph Pad Prism 8.3.1 (GraphPad Software Inc., San Diego, CA, USA). Quantitative analysis of lipase production were plotted using Graph-Pad Prism 8.3.1, Significance was accepted at $p<0.05$.

3 Results and discussion

3.1 Sample collection

Samples were collected from two oil refinery industries, Uma oil refinery industry, Kadi (North Gujarat), India & Mahakali oil refinery industry, Karannagar (North Gujarat), India.

3.2 Qualitative screening and isolation of lipolytic fungi

A total of 49 pure cultures were isolated from enriched soapstock samples. Among these, 19 cultures were fungal isolates. The fungal cultures were further subjected to the qualitative screening on tributyrin agar medium to select lipolytic strain. The clear zone around fungal isolates exhibited on tributyrine agar plates within 5 days indicates positive results. Range of 5.30 to 35.00 mm of clear zone of lipid hydrolysis was observed by lipase producing isolates. However, only six isolates that showed good zone of lipid hydrolysis in diameter ranged between 13.30 to 35.00 mm were selected for further study and results were represented (Table 1, Figure 1). Among the six fungal strains, 7F isolate was detected to produce maximum zone of hydrolysis (35.00 mm), whereas 12F isolate was produced minimum zone hydrolysis (13.30 mm) on 1% tributirine agar plates.

Table 1. Qualitative enzyme activity measure in clear zone diameter to colony diameter ratio of fungal isolates.

Sr.no	Fungal Isolates	Clear zone diameter ratio (mm)		
		Lipase activity	Cellulase Activity	Protease Activity
1	5F	16.00 mm	10.34 mm	08.68 mm
2	6F	19.70 mm	13.29 mm	11.90 mm
3	7F	35.00 mm	29.10 mm	15.00 mm
4	12F	13.30 mm	09.00 mm	07.39 mm
5	13F	20.00 mm	17.90 mm	14.17 mm
6	17F	16.30 mm	13.37 mm	10.00 mm

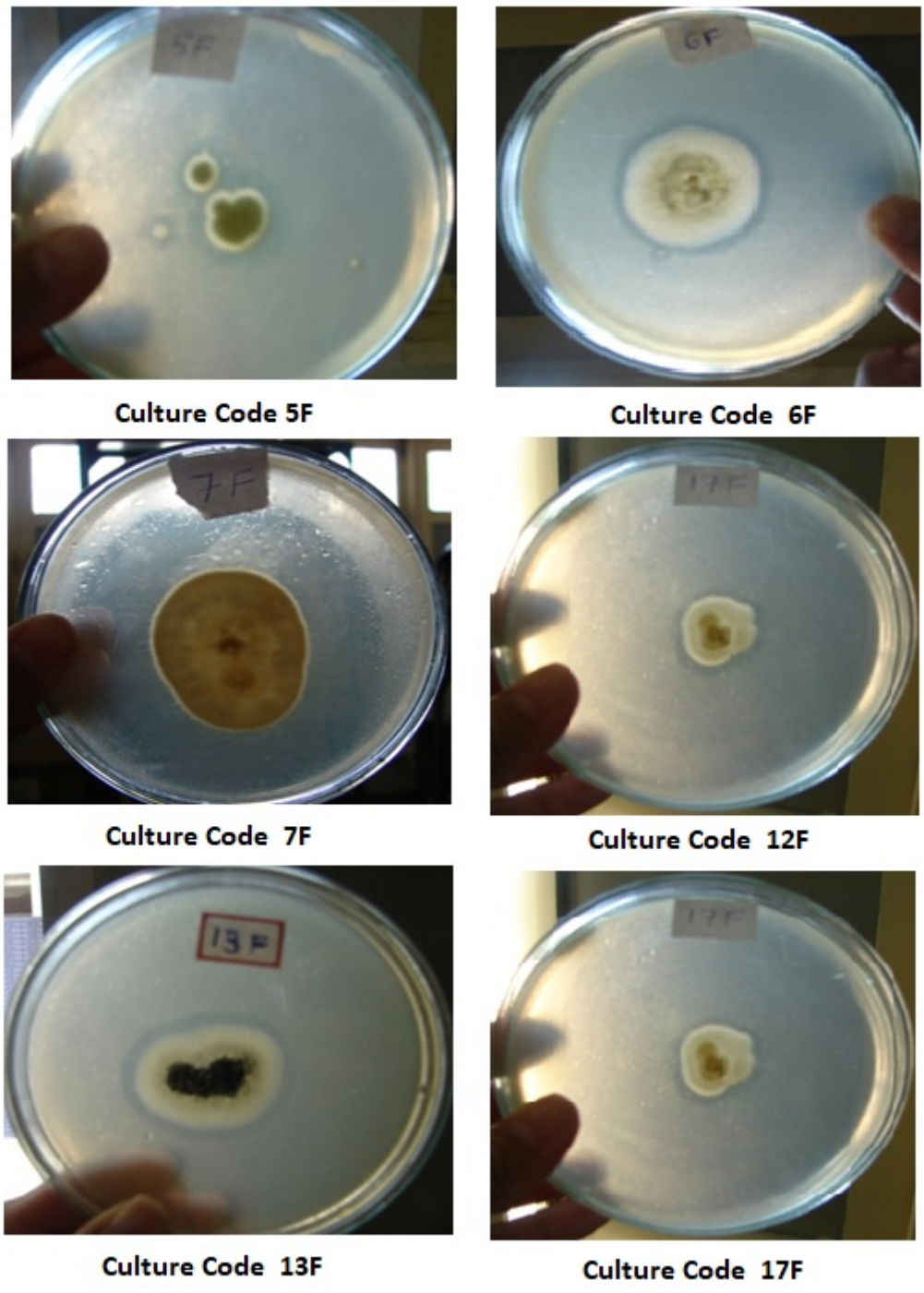

Fig 1. Qualitative screening of Lipase production by fungal cultures on 1% tributyrin agar plate

3.3 Quantitative analysis of Lipase enzyme under submerges fermentation

Agro-industrial wastes can easily be degraded by filamentous fungi, when synthesizing industrially important bio-compounds, such as lipolytic enzymes [34]. It is known that various fats, fatty acids, plant oils, triglycerides, ester-based detergents, and other substances are the best inducers of lipase synthesis by microorganisms and sources of carbon [35]. From earlier reported result *Aspergillus* species were used oils from agro-industrial waste (grape seed oil (GSO) and cotton seed oil (CSO)) to enhance lipase production further higher percentage of fatty acid esters (>80%), namely, soybean, olive, and GSO, promoted the highest lipase production [20]. Present study results supports the earlier report on use of cotton seed oil as carbon source to enhance lipase production by *Penicillium melinii* [35] and *Oospora lactis* fungi utilized cotton oil soapstock (oil richer) as carbon source and produced lipase enzyme [26]. *Fusarium* sp. are able to cause disease in plant growth stages [36,37]. In contrast they are good industrially important lipase producers. Growth of fungi was enhanced in the production medium supplemented with oil. High fungus mycelium, biomass and maximum crude lipase production were obtained on 4th day in submerged fermentation are comparable with the previous studies reported results on *Aspergillus* sp. strains [9]. The lipase producing strains *Aspergillus* spp. were more frequently present in palm oil mill effluent composts and were isolated from it [27].

Lipolytic strains were further quantitatively tested for crude lipase production by p- NPP as a substrate. In plate assay, short-listed six fungal isolates were further screen for lipase production by submerged fermentation. The enzyme activities were performed by all six selected isolates. The six selected fungal isolates (5F, 6F, 7F, 12F, 13F and 17F) were examined for lipase production after 120hrs cultivation in 100 mL liquid medium supplemented with 1% cotton seed oil as a substrate. In previously reported *F. solani* was isolated from decomposition of leaves in an aquatic environment, shown lipase production when utilizing Cotton oil as a carbon source in submerged condition [28]. Similar result was obtained; *Fusarium solani* 7F fungi produced lipase in presence of cotton seed oil in submerged fermentation. The activities ranged from 2.1 to 5.9 U/mL/min for lipase. From the observation, the 7F isolate exhibited the highest lipase activities, 5.9 U/mL/min than that the others. The lipase activities were shown by individual fungal isolates at different time period (Figure 2). 7F fungi showed maximum lipase activity 5.95 U/ml/min in submerged fermentation when incubated at 30^0C at pH 7. The activity of 7F, when compared to other fungi was highly significant ($p<0.0001$) at a time interval of 24hrs. Lipase activity (5.92 U/mL/min) of 7F was obtained maximum compare to lipase activity (5.72 U/mL/min) of *Mucor* [27].

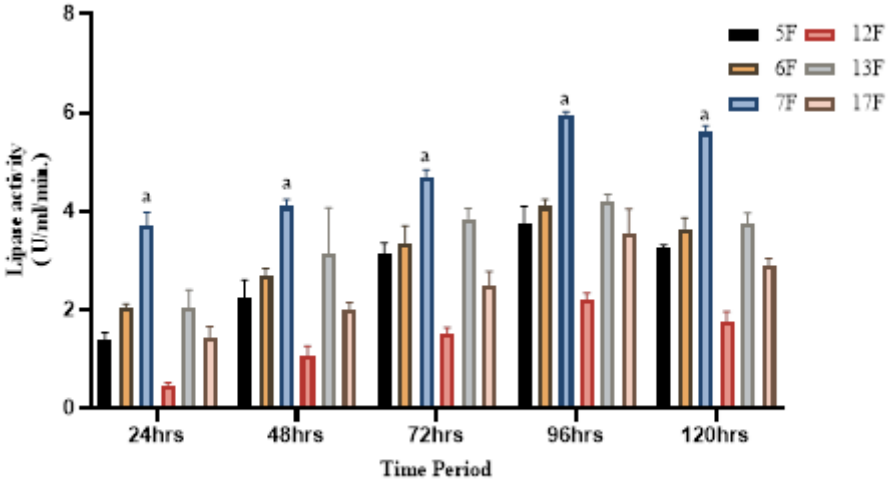

Fig 2. Time course study of different fungal isolates. ANOVA: p value = $p < 0.0001$, p value summary based on Tukey's multiple comparison tests = a highly significant at a time interval of 24hrs.

3.4 Enzyme profile

Fungi cultures were further screened for Cellulase and Protease activity by plate assay. Their cellulase and protease activity were detected by clear zone formation on CMC and milk agar plate respectively within 5 days and results were represented (Table 1). All six isolates showed positive result for cellulase and protease activity in plate assay. Among the six fungal strains, 7F isolate

was detected to produce maximum zone of hydrolysis (29.10 mm) on 1% carboxy- methyl cellulose agar plate, whereas 7F isolate was also detected to produce maximum zone hydrolysis (15.00 mm) on 1% skim milk agar plate.

3.5 Characterization of Fungal Isolates

The fungal isolates which showed the maximum zone of clearance for lipase production were subjected to further characterization and identification. The morphological and cultural characteristics of *Fusarium solani* were the same as those reported in previous work [36]. Growth characteristics of the *Fusarium solani* 7F isolate on PDA plate were white mycelia, which spread very fast, hyphae branched and septate. *Fusarium solani* 7F grows radials and spores produced. Yellowish pigments appear on the back of the plate. *Penicillium griseofulvum* 5F fungi isolate grow slowly, mycelia green cottony and back of plate yellow pigment. *Aspergillus flavus* 6F culture mycelium spread fast, mycelia white initially after three days incubation macro spore turn green and back of plate no pigment. Zone observed in two parts white to green centre. *Aspergillus niger* 12F fungus moderately grows on PDA, mycelia white cottony and no pigment observed. *Aspergillus niger* 13F isolate growth very fast, mycelia white initially on aging macro spore turn black and back of plate no pigment. Zone observed in two parts white to black centre. *Aspergillus terreus* 17F culture growth was fast, mycelia initially white after three days incubation macro spore turns brown. Zone observed in two parts white to brown centre. Potent fungi culture was identified by morphological, cultural and molecular characteristics culture 5F, 6F, 7F, 12F, 13F and 17F were identified as *Penicillium griseofulvum*, *Aspergillus flavus*, *Fusarium solani*, *Aspergillus niger*, *Aspergillus niger* and *Aspergillus terreus* respectively (Figure 3). The sequence of the gene deposited at the NCBI repository is accessible by Accession numbers (Table 2). The Phylogenetic tree was created with maximum likelihood methods and Tamura-Nei model shows evolutionary history of deposited sequence with other aligned sequence. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated Taxa clustered together in the bootstrap test (500 replicates) was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA X (Figure 4). Reported fungal strains such as *Aspergillus flavus*, Aspergillus niger and *Aspergillus terreus* showed the degradation of crude oil due to the production of extracellular enzymes [38].

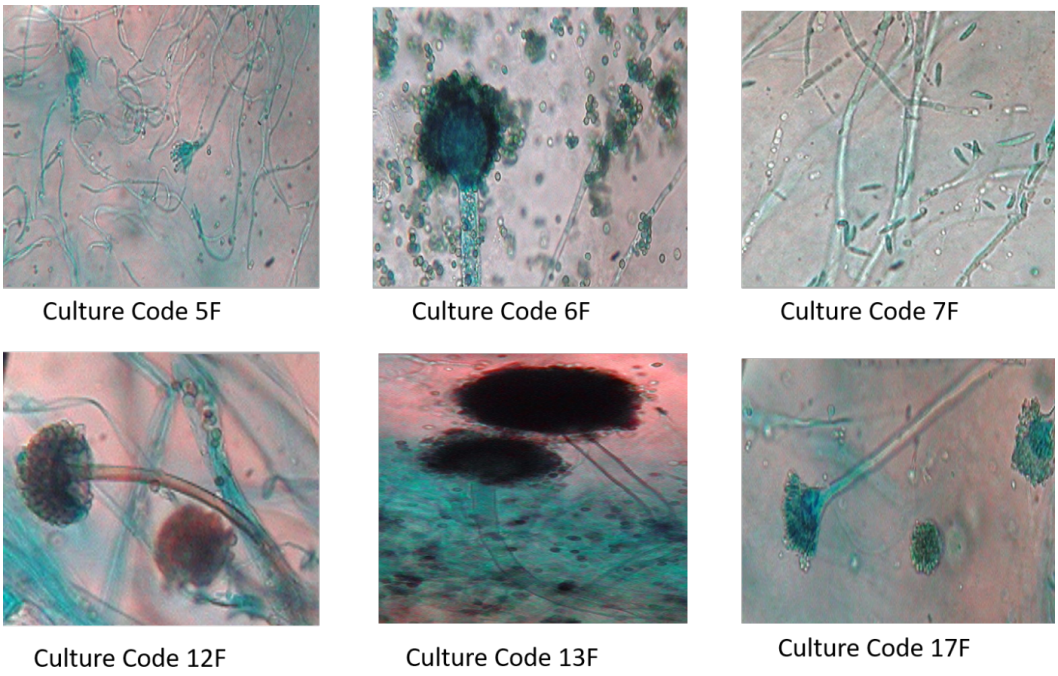

Fig 3. Microscopic observation of isolated fungi

Table 2. Accession number of isolated Fungi

Sr.no	Culture code	Accession number	Organism
1	5F	MN749934	*Penicillium griseofulvum*
2	6F	MN750585	*Aspergillus flavus*
3	7F	MH571778	*Fusarium solani*
4	12F	MN750583	*Aspergillus niger*
5	13F	MH571779	*Aspergillus niger*
6	17F	MN750586	*Aspergillus terreus*

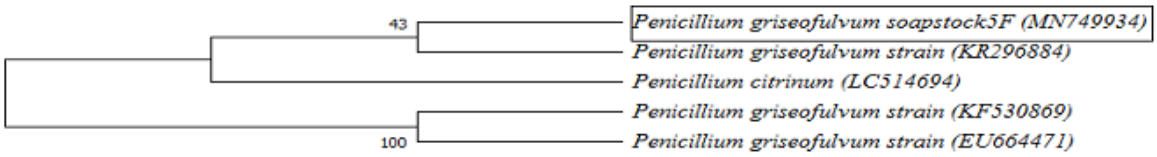

Figure 4a. Phylogenetic tree of *Penicillium griseofulvum soapstock5F (MN749934)*

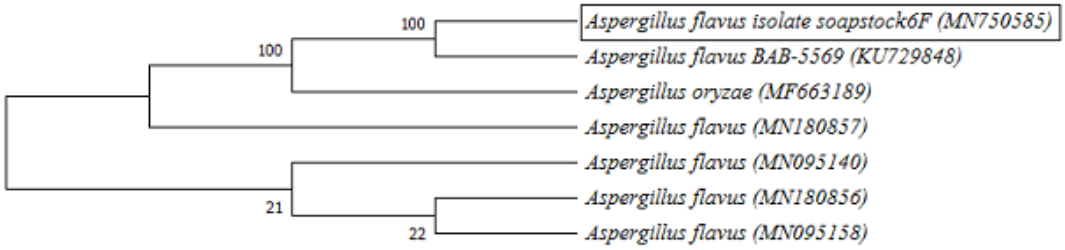

Figure 4b. Phylogenetic tree of *Aspergillus flavus soapstock 6F (MN750585)*

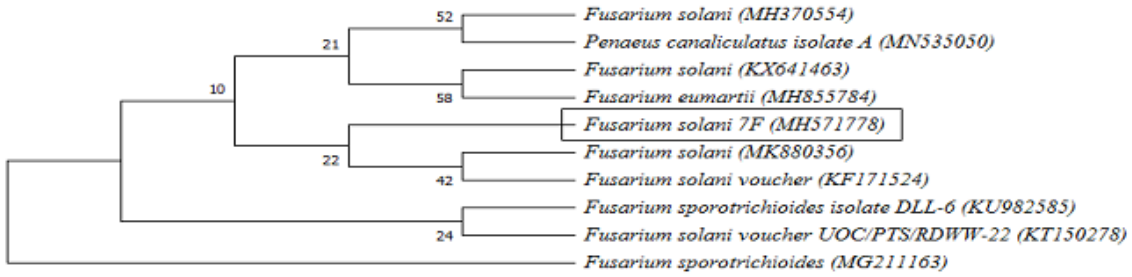

Figure 4c. Phylogenetic tree of *Fusarium solani 7F (MH571778)*

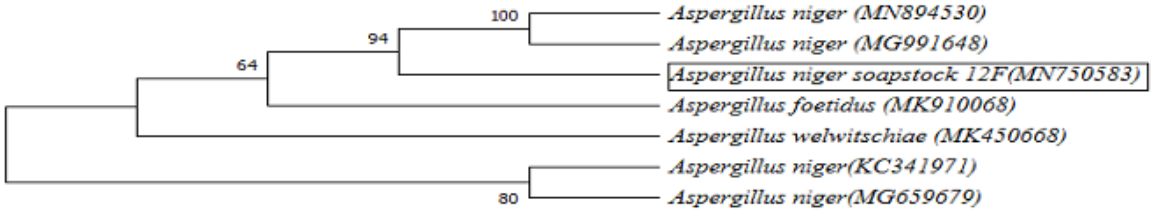

Figure 4d. Phylogenetic tree of *Aspergillus niger soapstock 12F (MN750583)*

Fig 4.

4 Conclusion

Cotton seed soapstock samples were collected from the oil refinery industry for the isolation & screening extracellular lipase producing micro-organisms. 49 isolates including bacteria & fungi were screened. The isolates showed highest lipase production in plate assays were further quantitatively tested for production of lipase by pNPP as substrate assay. The isolated micro-organisms have higher ability to produce lipase enzyme. In further studies pilot scale lipase production and its purification studies will be conducted. Enzyme derived from *Fusarium solani* 7F is further used in the microbial degradation of soapstock and production of biosurfactant. The identified cultures were deposited in the NCBI culture collection center with accession number.

Acknowledgements

The authors are thankful to the Department of Biotechnology, Pramukh Swami Science and H.D. Patel Arts College, KSV, I for providing facilities to carry out research. The authors also wish to express thanks to Gujarat State Biotechnology Mission at Gandhinagar, India for the valuable advices in the identification of the Fungi isolates.

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Proximate Analysis and Evolution of Energy Value from Leaves and Stem of Sword Fern: Nephrolepis exaltata

Article *in* International Research Journal of Pure and Applied Chemistry · October 2020

DOI: 10.9734/IRJPAC/2020/v21i1730261

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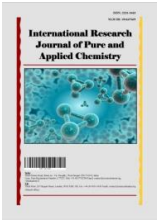

International Research Journal of Pure & Applied Chemistry

21(17): 10-17, 2020; Article no.IRJPAC.61303
ISSN: 2231-3443, NLM ID: 101647669

Proximate Analysis and Evolution of Energy Value from Leaves and Stem of Sword Fern: *Nephrolepis exaltata*

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Authors' contributions

This work was carried out in collaboration among all authors. Author DKS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors RSD and KRS managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IRJPAC/2020/v21i1730261
Editor(s):
(1) Dr. Hao-Yang Wang, Shanghai Institute of Organic Chemistry, China.
Reviewers:
(1) Ayobami Omozemoje Aigberua, Niger Delta University, Nigeria.
(2) Syed Mubashar Sabir, University of Poonch Rawalakot, Pakistan.
Complete Peer review History: http://www.sdiarticle4.com/review-history/61303

Original Research Article

Received 15 July 2020
Accepted 22 September 2020
Published 05 October 2020

ABSTRACT

The purpose of present study was to prognosticate the amount of each nutrient in the Arial parts of sword fern "*Nephrolepis exaltata*". The study was conducted at Sarva Vidhyalaya Campus, Kadi, Gujarat (India) during the period of December-2019 to March-2020. The prognostication of nutritive component including ash%, moisture%, Crude Protein%, Crude Fat%, Crude fibers% and Carbohydrate was taken from AOAC and Standard Lowry method for estimation of protein. The proximate analysis indicates the monumental presence of crude protein and carbohydrates, moderate presence of crude lipid and fibers. The ash content was 8.65% and 12.42% in leaves and stem respectively, which manifests the existence of inorganic minerals. The Crude protein was recorded as 21.25% and 30.0% in leaves and stems respectively, whereas the crude lipid was 8.41% and 1.47% respectively. The leaves and stems were found to be a good source of energy value with 359.766 Kcal/100 gm and 313.266 Kcal/100 gm respectively.

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Keywords: Nephrolepis exaltata; proximate analysis; Lowry method; energy value; AOAC (Association of Official Analytical Chemists); pteridophyte; vascular plants.

1. INTRODUCTION

In 1860, Henneberg and Stohmann developed a method for quantitative analysis for discriminate the different macronutrients in feed, was explored as proximate analysis. Proximate analysis is not wholly nutrient analysis, rather it is a partitioning of both nutrients and non-nutrients into categories based on common chemical properties. In Proximate Analysis, based on the chemical properties, the compounds in a feed are partitioned into six different categories. They include: moisture, ash, crude protein, crude lipid, crude fibre, nitrogen-free extracts (digestible carbohydrates) [1].

To acquire the approximate amounts of substances within a material the Proximate analysis can be the best scientific inquiry. This method is traditionally utilized in contrasting scientific fields to study diverse materials such as animal feed, coal, and bio-fuels etc. The process is quite intricate involving extraction of one material through miscellaneous solvents or extraction of different materials through single solvent. The hazard potential of chemicals can be determined by proximate analysis. This information can be used to create quality controls for various materials which ensure that they are healthy enough to be consumed by humans or animals [2].

Proximate analysis of plant is the best way to ensure the composition of essential biomolecules such as protein, fats, and carbohydrates etc. In human food products, nutrient levels such as proteins, fats and carbohydrates are often determined [3].

Desert area is the most engrossing part of the mother earth and the point of origin for many plants' species, from ferns to modern dicots. Pteridophytes including ferns and fern-allies are non-flowering, vascular and spore bearing plants. They are ostentatious plants of the earth's vegetation and from evolutionary perspective they are important as they show the evolution of vascular system and exposure of seed habit in the plants. They are abundant in moist tropical and temperate forests and grow in different intermediate regions from sea level to the highest mountains [4]. Ferns are vascular plants that do not produce seeds. *Nephrolepis exaltata*, an epiphytic and terrestrial fern was taken for current study. There are around thirty species in the genus *Nephrolepis* [5]. *Nephrolepis exaltata* is a customarily found pteridophyte and vocalized as "Boston fern" or "sword fern" which is classified in the family *Nephrolepidaceae* [6].

Nephrolepis is easily elucidated and morphologically quite sui generis plant; the stem is a minuscule erect stock with closely tasseled fronds. It has a willowy lateral branch bearing numerous roots and occasional buds which develops into new plants. The genus *Nephrolepis* belongs to Family Nephrolepidaceae, which includes 35 Genera and 303 accepted taxa overall with 2 Subspecies. The order of plant is 'Polypodiales' [7]. The plants of this order are also known for their resistivity in desert area.

Different studies have established the presence of antioxidant activity and also anti-inflammatory property in many species of *Nephrolepis* [8]. Some studies on mice shows that the extract preparation of *Nephrolepis biserrata* given at 100 mg/kg within an hour interval can reduce the level of inflammation. The leaves of *Nephrolepis biserrata* are used for treatment of blisters, boils, abscesses, sores, abdominal pains, wounds and cuts [9].

The result of the proximate composition for some plants showed that the seed oil possessed good physicochemical properties which are the main component of edible oil. As such, this type of plant parts could be utilized successfully as a source of edible oil for human consumption and for industrial applications [10].

Despite the progress made in research of *Nephrolepis exaltata* during the past decades, some phytochemical constituents of the plants are yet to be discovered. As such, the plant yet retains further potential to provide the master stroke for resolving some of the globe's major health problems. However, this study seeks to examine the potential of *Nephrolepis exaltata* for nutritional use. Although some studies revealed that the aqueous extract of fern *Nephrolepis exaltata* may be cardiotoxic to some Arthropods like cockroaches in a dose-dependent manner. Some of unidentified terpene found in aqueous extract of *Nephrolepis exaltata* could play a part for the cardiotoxic effect of in cockroaches [11]. Some studies revealed facts about the allergic effects of Boston fern *Nephrolepis exaltata*,

which is commonly vocalized as 'Bostoniensis' [12]. Proximate analysis is most dependable strategy to find nutritional composition of numerous plants and their parts. Some proximate analysis methods have been used to interpret the nutrition relationship between monocots Maize and sorghum [13].

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

The fresh plants were collected from a local surrounding in Kadi (23.2973°N, 72.3302°E) during the month of December 2019. Identification and authentication of the plants was done by Taxonomist and Associate Professor, Dr. K.J Bhatt at the Department of Biology, Pramukh Swami Science and H.D Patel Arts College, Kadi.

2.2 Processing of Plant Materials

Nephrolepis exaltata plant leaves and stem were air dried at 28°C to 40°C for 30 days. Thereafter, samples were milled into fine powder using electric blender. This was done in order to increase the surface area of sample for improved solvent penetration of cells and enhanced extraction of secondary metabolites.

Although fresh and dried samples can be applied in plant extraction studies, dried samples are mostly preferred when considering the time needed for experimental design. Vongsak compared the extraction of fresh and dried plant parts and concluded that fresh samples are fragile, with tendency to deteriorate faster than dried samples, hence extraction is faster in dried samples [14].

2.3 Proximate Analysis

The AOAC methods were used to determine proximate analysis of all plant samples. Powdered plant material was used for analysis of % Ash, % moisture, Crude protein, Crude lipid, Carbohydrates and elemental composition of the selected plant species.

The Proximate analysis was carried out by following scheme-

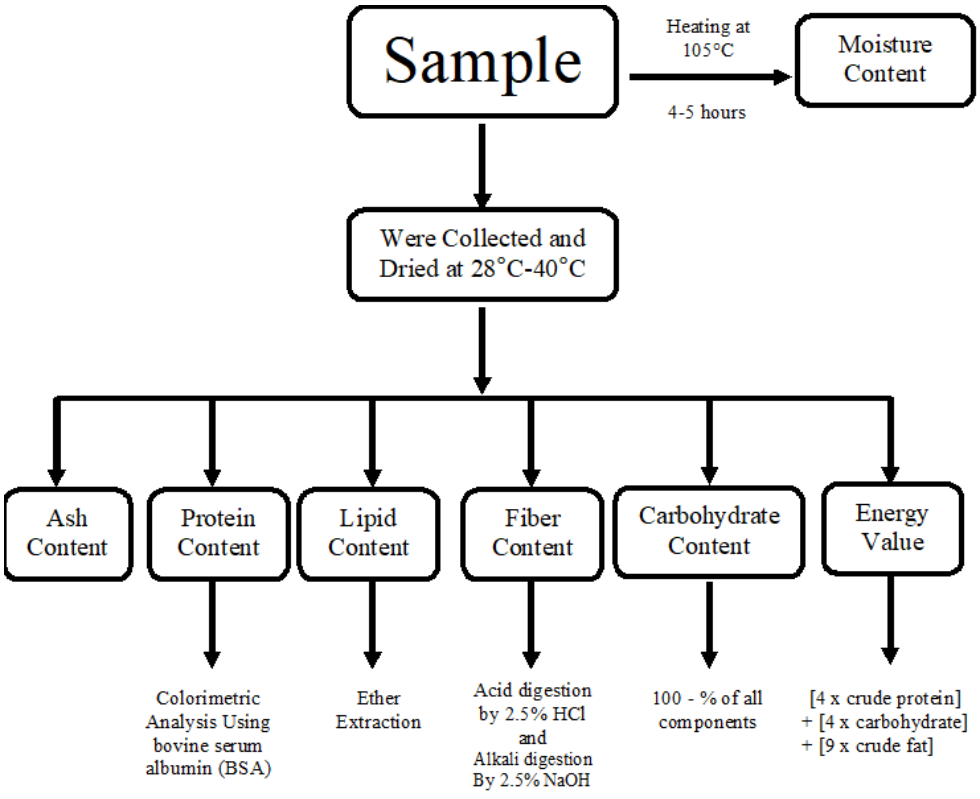

Fig. 1. Scheme for proximate analysis of *Nephrolepis exaltata*

2.3.1 Determination of % ash

Ash was determined according to AOAC (2000) method. At 660°C a clean crucible was heated in a muffle furnace for an hour, then in a desiccator it was cooled and weighed as (W_1). For the determination of ash content, 10 g dry sample was taken in the crucible. The sample was scorched over the burner with the help a blowpipe. Thereafter, the crucible containing sample was heated at 550°C for 6-8 hours in a muffle furnace. When6 the embers left in crucible after the complete ignition the furnace was turnoff. After cooling the crucible at room temperature, it was weighed as (W_2). Percent ash was calculated as follow.

$$\%\text{Ash} = \frac{Wt.of\ Ash\ (W_2 - W_1)}{wt.of\ sample} \times 100$$

2.3.2 Determination of the moisture

Standard AOAC method was followed to deduce the Moisture contents. A dry empty pre-weight (W_1) clean petri-dish (with lid) was filled with 1 gram of sample. Samples were oven-dried at 105°C for 4-5 hours until constant weight was obtained. After which Sample was removed and placed in desiccator for 30 minutes in order to cool it. After cooling the dish, the final weight (W_2) was measured was weighted. Percent was calculated as follows:

$$\%\text{Moisture} = \frac{Wt.of\ residue\ (W_2 - W_1)}{wt.of\ sample} \times 100$$

2.3.3 Determination of proteins

The content of proteins can be ascertained using different methods. From a comparative analysis to determine total protein using the Kjeldahl method and several spectrophotometric methods in a number of different samples, it was concluded that the Lowry method showed the lowest variance using BSA (Bovine Serum Albumin) as standard protein for precise absorption [15].

Due to simplicity and availability of reagents The Lowry method has been widely used for protein determination. However, besides aromatic amino acids, a wide range of other compounds react with the Folin–Ciocalteu reagent [16]. The modified Lowry protein measurement was conducted according to the method described by Hartree in 1972 [17].

2.3.3.1 Proteins extraction

For the estimation of protein in different plant parts, samples were homogenized separately. About 10% trichloroacetic acid (TCA) was applied to the plants since TCA has the property to dissolve macromolecules such as proteins, DNA, and RNA so it is widely used in biochemistry for the precipitation. After preparation of homogenized solution, centrifugation followed at 5000 rpm for 10 minutes. The Supernatant liquid portion was discarded and pellets containing the protein were recovered. Pellets were again suspended in 5 ml of 10% cold TCA and recentrifuged for 10 minutes. Supernatant was again discarded and the precipitate was dissolved in 10 ml of 0.1 N NaOH. The solution was used for protein estimation.

2.3.3.2 Quantitative estimation of proteins

Using the protocol of Lowry *et al.*, 1951, total protein content was estimated in about 1 ml extract.

1. For preparation of a stock solution of bovine serum albumin (1 mg/ml), 1 N NaOH was used.
2. The five-working solutions of different concentrations (2, 4, 6, 8 and 10 ml) from the working standard solution were taken in series of test tubes.
3. In another set of test tubes 0.1 ml and 0.2 ml of the sample extracts were taken and the volume was made up to 1 ml in all the test tubes using distilled water.
4. A mixture solution (A) was prepared by mixing 50 ml of 2% Na_2CO_3 in 0.1 N NaOH and 1 ml of 0.5% $CuSO_4$. $5H_2O$ in 1% sodium potassium Tartrate.
5. The 5 ml prepared mixture (A) was added to each test sample at room temperature.
6. The prepared test samples were left undisturbed for a period of 10 min.
7. Subsequently, A mixture of 0.5 ml of distilled water mixed with 0.5 ml FC reagent B was added to each mixture tube at room temperature (25°C) for 30 minutes until the blue color developed.
8. The Equip-Tronics colorimeter (µP based Inbuilt Filter-Model No-WQ-652) was adjusted at wavelength of 720 nm and set at 100% transmittance using blank before taking the readings of the standard and the test samples respectively.
9. Five replicates were examined in each case and their mean values were recorded.
10. A regression curve was worked out of various concentrations of the standard

solutions against their respective absorbance in accordance to the Beer's law.

2.3.4 Determination of crude lipid (fat content)

There are a variety of test methods for fats, but generally the method can be broken down into "crude" or total fats. "Crude" methods involve dissolving the material in a solvent such as ether or hexane followed by solvent evaporation. The material that remains is called "crude fat". In this case, ether was used as solvent to dissolve the lipid content.

1. A thimble with dry sample was prepared and its weight recorded as W_1.
2. The extraction was done using Soxhlet extractor with diethyl ether as solvent.
3. The thimble was placed into a thimble holder and the holder was clipped.
4. Then after a sufficient amount (one glass reclaiming tube full - approximately 40 ml) of diethyl ether was poured into RBF (Round bottom flask) of sohxlet apparatus, so the plant material in the thimble could be extracted.
5. The RBF was coupled to the extractor with the ring clamp tightly adjusted.
6. The temperature was set to 40^{o}C in heating element.
7. The heater switch, the main power switch, and the condenser water were turned on.
8. As the ether solvent boiled, glassware was inspected for leaks so as to ensure that there were no volumetric losses.
9. Extraction was carried out using standard methods for 16 hours at Low setting (condensation rate of 2 to 3 drops per sec).
10. After extraction, the temperature of heating element was lowered down by shutting down the power and water supply and allowed the ether to drain out of the thimbles (about 30 min).
11. Remove the thimble from the holder, and rinse the holder with small portions of diethyl ether from the wash bottle.
12. The extract was transferred into a pre-weighed beaker (W_2) for further evaporation at room temperature.
13. Room temperature drying was employed in order to avoid possible explosion from oven-induced drying of the ether solvent.
14. The weight of beaker and residue (W_3) was recorded on completion of the drying process.

    Note: Excessive drying may oxidize the fat and give high results.
15. The remaining residue in thimble was used for fiber analysis.

$$\%\text{Crude Lipid} = \frac{Wt.of\ extract\ (W_3 - W_2)}{wt.of\ sample\ (W_1)} \times 100$$

2.3.5 Determination of crude fibers

Crude fiber was determined by acid and alkali digestion method using fiber tec apparatus by following AOAC (2000) [18].

1. The weighed (W1) thimble residue samples were first digested with acid and then with alkali.
2. The sample was transferred into a clean glass beaker a clear beaker and 100 ml of 2.5% HCl was added to it.
3. The mixture was boiled with stirring for about half an hour. It was then drained into the beaker.
4. The fiber residue was again digested in 2.5% NaOH, similar to the acid digestion process.
5. The residue collected was transferred to a pre-weighted (W_2) dried crucible to remove the moisture.
6. The crucible was then kept in furnace for red dull heat till the formation of white and grey ash. The crucible was cooled in desiccators and weighed again. (W_3)
7. The loss in weight of the dry residue upon ignition was taken as the amount of crude fiber. Percent crude fiber was calculated as follows-

$$\%\text{Crude Fiber} = \frac{Wt.of\ dry\ residue\ (W_3 - W_2)}{wt.of\ sample\ (W_1)} \times F \times 100$$

Where,

F = Value of crude fat, W_1 = Weight of Sample, W_2 = Weight of dry crucible, W_3 = Weight of crucible after heating

2.3.6 Determination of carbohydrate contents

Carbohydrates were determined by subtracting the weights of protein fats, crude fibers, ash, and moisture contents from 100.

$$\text{TCH (\%)} = 100 - \%(\text{CP} + \text{A} + \text{CF} + \text{M})$$

2.3.7 Determination of energy value

Carbohydrates give average gross energy values of 4.2 kcal or 17.6 kJ per gram, fat gives 9.4 kcal, or 39.4 kJ per gram and protein gives 5.65 kcal or 23.7 kJ per gram. So, the energy values were

estimated by calculation method using the formula [19],

Energy value (g/100g) = [5.65 x crude protein] + [4.2 x carbohydrate] + [9.4 x crude fat]

3. RESULTS AND DISCUSSION

Data in Table 1 represents the "Proximate Analysis and Energy Value" of *Nephrolepis exaltata*. The dry sample of leaves was light green in color with specific agreeable odor whereas the dry sample of stem was subtle colored. The percentage yield of Ash content was found to be higher in stem, which indicates the presence of high mineral content. The moisture content in leaves and stem was found to be very high, ranging between 21.78% in leaves and 24.83% in stem. Apart from moisture the major chemical constituent found was carbohydrate, recording a maximum content of maximum content 38.32% in leaves and 30.94% in Stem. Crude protein content was estimated as 21.25% in leaves and 30.00% in stem, while crude fat (Lipid) content observed was 8.41% in leaves and 1.47% in stem. The crude fiber content with maximum concentration 1.59% was determined in leaves while minimum amount 0.34% was found in stem.

Table 1. Proximate analysis and energy value of *Nephrolepis exaltata* plant leaves and stem (% of dry samples)

Composition	*Nephrolepis exaltata* (Leaves)	*Nephrolepis exaltata* (Stem)
Ash %	8.65%	12.42%
Moisture %	21.78%	24.83%
Crude Protein %	21.25%	30.00%
Crude Lipid %	8.41%	1.47%
Crude Fiber %	1.59%	0.34%
Carbohydrate %	38.32%	30.94%
Energy Value (Kcal/100 gm)	359.766	313.266

The moisture content was observed 21.78% in leaves and 24.83% in stem respectively; The result was compared favorably with that of Oloyede *et al.* (2012) and Adebiyi el at. (2016) who worked on the *Nephrolepis furcans* and *Nephrolepis cordifolia* (L) in Nigeria respectively [20,21]. This indicates that the amount of moisture varies from species to species and from geographical locations. Low moisture content promotes shelf life of plant and hamper the growth of microorganism. The ash content gives an indication regarding the amount of minerals present in a particular sample, which are important in many biochemical reactions functioning as co-enzyme and aid physiological functioning of major metabolic processes in the body. It contains inorganic material of the plant because ashing destroys all the organic material present in the sample. High ash content in any food substance is an indication of high mineral content [22].

In contrast with other species, *Nephrolepis exaltata* do have higher protein level. The results of the protein analysis observed in this study; is almost similar what was previously reported for *Nephrolepis exaltata* by Johnson M *el at.* (2016). The relative positions of the protein bands were studied for *Nephrolepis exaltata* (L.) through SDS-PAGE and the plant showed maximum number of protein bands [23]. The Fiber content was found in small extent as it was observed in *Nephrolepis furcans* by Oloyede *et at.* 2012, which revealed the lower presence of Fat-free organic substances [20]. The result of the carbohydrate analyses of the plant is reported higher than the other species of plant which shows the food storage capability.

From the above results, it was shown that *Nephrolepis exaltata* has lesser amount of nutrients such as carbohydrates and crude fiber, but it had been found major amount of carbohydrate and protein in it, that determine nutritional value of the fern.

4. CONCLUSION

In conclusion the proximate analysis of *Nephrolepis exaltata* showed that the Sword fern is a good source of essential nutrients like protein, carbohydrate, fat, fibers. Protein analysis by Lowry method indicated that sword fern might be a rich source of isolatable protein. The plant has high ash content, thereby indicating the presence of minerals and Inorganic components. It can be inferred that due to the presence of primary constituents the several solvent extracts from Sword fern can be utilized in different sectors. The presence of different phytochemicals, anti-microbial, anti-fungal potential can also be stabilized. This study suggests that *Nephrolepis exaltata* can be a promising material for pharmaceutical application.

ACKNOWLEDGEMENTS

We thank Dr. A. S. Gor, Principal, PSSHDA and Director, HVPG, kadi and Dr. Minal Trivedi, Principal, HVPGR, Kadi for their support and

Encouragement. We express our sincere thanks to HVPGR for providing laboratory assistance and infrastructural facilities

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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DOI: 10.4172/2472-0992.1000112

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/61303

RJC

RASĀYAN *J. Chem.*
Vol. 13 | No. 1 |333 - 338| January - March | 2020
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

SYNTHESIS AND CHARACTERIZATION OF CROSS-LINKED TRI-POLYMERS OF POLY ACRYLIC ACID AS WATER THICKENING AGENTS

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ABSTRACT

A cross-linked poly acrylic acid tri-polymer is successfully synthesized by batch charging polymerization of poly allyl tris buffer, di-vinyl benzene and acrylic acid and this new method is profitable in terms of reducing the toxicity, faster, purity and cheaper than available methods. The morphological structure of the synthesized polymer is studied by SEM and the characteristic functional groups of the synthesized polymers are identified using FTIR. TGA and DSC techniques were employed to study their thermal characterization.

Keywords: Poly allyl tris buffer, Di-vinyl Benzene, Acrylic Acid, Water-absorbing Agent, Copolymer Composition

INTRODUCTION

Co-polymers of the carboxylic monomer for instance acrylic acid and 0.1 - 1.0 by weight cross-linker like poly allyl tris buffer are gel-like polymers. These types of polymers are unsolvable in aqueous medium and other organic solvents. They are particularly in the type of their salts; suck up great quantities of water or other solvents with subsequent a numerous times raise in volume. The carboxylic polymers produced when a carboxylic monomer is copolymerized with a poly alkenyl polyether of a polyhydric alcohol. It is containing two or more alkenyl ether grouping per molecule.[1-2]

These carboxylic polymers are used in various fields such as thickeners, suspending agents and stabilizers.[3-6] Polymers of a carboxylic acid monomer and acrylic esters having aliphatic long-chain containing at least 10 - 30 carbon atoms can be used as thickeners when they are neutralized. They have superior resistance to reducing in viscosity when salt added thereto.[7]

Excessively spongy polymers are crossly linked polymer have a network like a sequence without dissolving and can retain just as grasp the vast volume of water in the swollen arrangement. The retained fluid is scarcely removable still under certain powers.[8]

The most frequently working synthetic polymers are evenly cross-linked carboxylic polymers primed from unsaturated carboxylic acid-containing monomers such as acrylic acid and maleic acid and anhydride. The cross-linked with unsaturated di-esters, di-vinyl benzene and others of carboxylic monomers are difficult to control in this polymerization. The various processes for synthesizing the higher carboxylic polymers are described in different Patents.[9-11] pH-sensitive cross-linked AA/Gelatin hydrogels produced by free-radical polymerization in the presence of ammonium persulfate initiator and ethylene glycol di methacrylate cross-linking agent. Different feed ratios of acrylic acid, gelatin, and EGDMA were used to investigate the effect of monomer, polymer, and degree of cross-linking on swelling and release pattern of the model drug.[13] Zinc oxide (ZnO) Nano composite hydrogels are

http://dx.doi.org/10.31788/RJC.2020.1315526

synthesized with free-radical polymerization in methanol using ammonium persulphate as the initiator at 60 ºC temperature and N, N'- methylene bis acrylamide (MBA) as a crosslinker.[14] The high swelling polymer hydrogel from epichlorohydrin with hydrolyzed polyacrylonitrile is synthesized and the swelling properties are identified. The effect of temperature and concentration is also analyzed. [15] Gold nanoparticles are prepared with poly (acrylic acid) and cysteine, it is characterized by techniques. [16]
The present research studies have employed a new synthetic method for the preparation of the tri polymers of AA.

EXPERIMENTAL

For this research work allyl tris buffer, di-vinyl benzene, acrylic acid, ethanol and potassium thiosulphate all these reagents are of laboratory reagent grade. Basic glassware such as three-neck round bottom flask and mechanical stirrer and FT-IR spectrophotometer (Bruker alpha) were used.
A nitrogen gas inlet tube and mechanical stirrer are built-in with a three-necked round bottom flask of 1000 ml capacity. Exactly 600 ml of the solvent mixture is transferred into the flask and add the two small pieces of porcelain chips. Now gradually heat the solvent mixture to 60-65ºC temperature and then pass the nitrogen gas to sparge the flask contents to oust the air. The 5 experimental runs (3.1-3.5) are set using varied amounts of allyl tris buffer (1, 0.8, 0.6, 0.4, 0.2 g) and relevant of acrylic acid and di-vinyl benzene added. At the same time, 1 g of an initiator potassium thiosulphate ($K_2S_2O_8$) is added to initiate the polymerization and temperature is expanded by 4°C/min to reach 80°C and this temperature is maintained for 1.5 h. During this heating period, the gelatinized polymer starts to produce within 50 minutes. The produced strong gel is dried for 24 hours. The dried solid is then used for further investigation. Then weigh 0.5 g solid polymer in a beaker to make suspense solution in 30 ml water overnight. Then remove the excess amount of water from a beaker and the swollen product is neutralized with saturated NaOH at about 7.0 pH. The reaction mixtures employed are given in Table-1.

Table-1: Description of the Amount of Reagents

Experiment	3.1	3.2	3.3	3.4	3.5
Chemicals	Amount	Amount	Amount	Amount	Amount
Allyl tris buffer	1 g	0.8 g	0.6 g	0.4 g	0.2 g
Di-vinyl benzene	39 g	39.1 g	39.2 g	39.3 g	39.4 g
Acrylic acid	60 g	60.1 g	60.2 g	60.3 g	60.4 g
Ini.– $K_2S_2O_8$	1 g	1 g	1 g	1 g	1 g
Sol.-H_2O+C_2H_5OH	490 + 10 ml	490 + 10 ml	490 + 10 ml	490 + 10 ml	490 + 10 ml
Neut. - NaOH	7 pH	7 pH	7 pH	7 pH	7 pH

RESULTS AND DISCUSSION

The polymer thickeners are identified by Fourier Transform Infrared (BROOKER FT-IR) Spectrophotometer using their KBr pellets and the representative IR spectrum of the polymer 3.1 is presented in Fig.-1. The spectra show that the main characteristic peaks are at 2924 $cm^{-1}$and 2855 cm^{-1}. These are exhibited for the asymmetric stretching vibration of (-C=C-H Stretching) and asymmetric stretching vibration of N-H of the secondary amine group. The strong peak at 1696 cm^{-1} confirmed –C=O stretching of intermolecular hydrogen bonding of acrylic acid. Furthermore, the IR band at 1652 (merged, weak) cm^{-1} is owing to the characteristic stretching vibration of -C=C bond. The peak at 1396 cm^{-1} is accredited to O-H bending vibration. The band at 1156 cm^{-1} is recognized for –C - O stretching of ether bending vibration.
The thermo gravimetric analysis (TGA) of the polymers was run using TG (Perkin Elmer TGA 8000 Thermo Gravimetric Analyzer). The thermograms indicate that degradation of a polymer starts around 150°C and degradation completed above 200°C temperature. Thus, it can be concluded that the prepared polymer thickeners are thermally stable up to 150°C and few are up to 250°C. The canyons (converse peaks) are due to the fact that polymer has started to degrade as the impurity could be adhered in the form of aqueous phase or hydrocarbons and heat is liberated during degradation.
The cross-linking is also characterized by DSC analysis, as shown in Fig.-3. DSC of cross-linked thickener polymer indicates that a weight loss occurs within two stages.

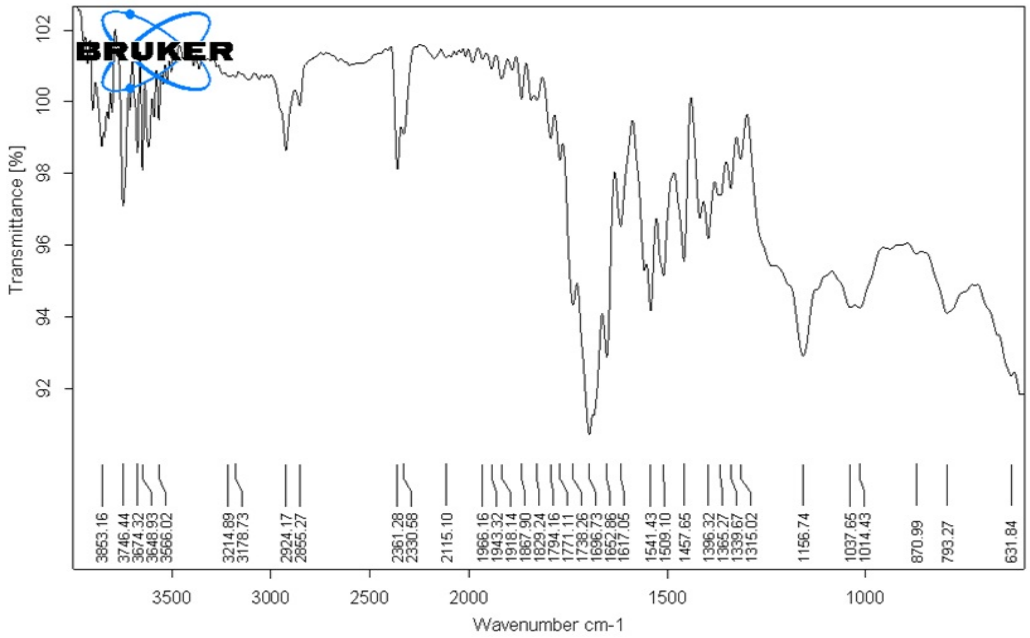

Fig.-1: FT-IR of Cross-linked Tri-polymer

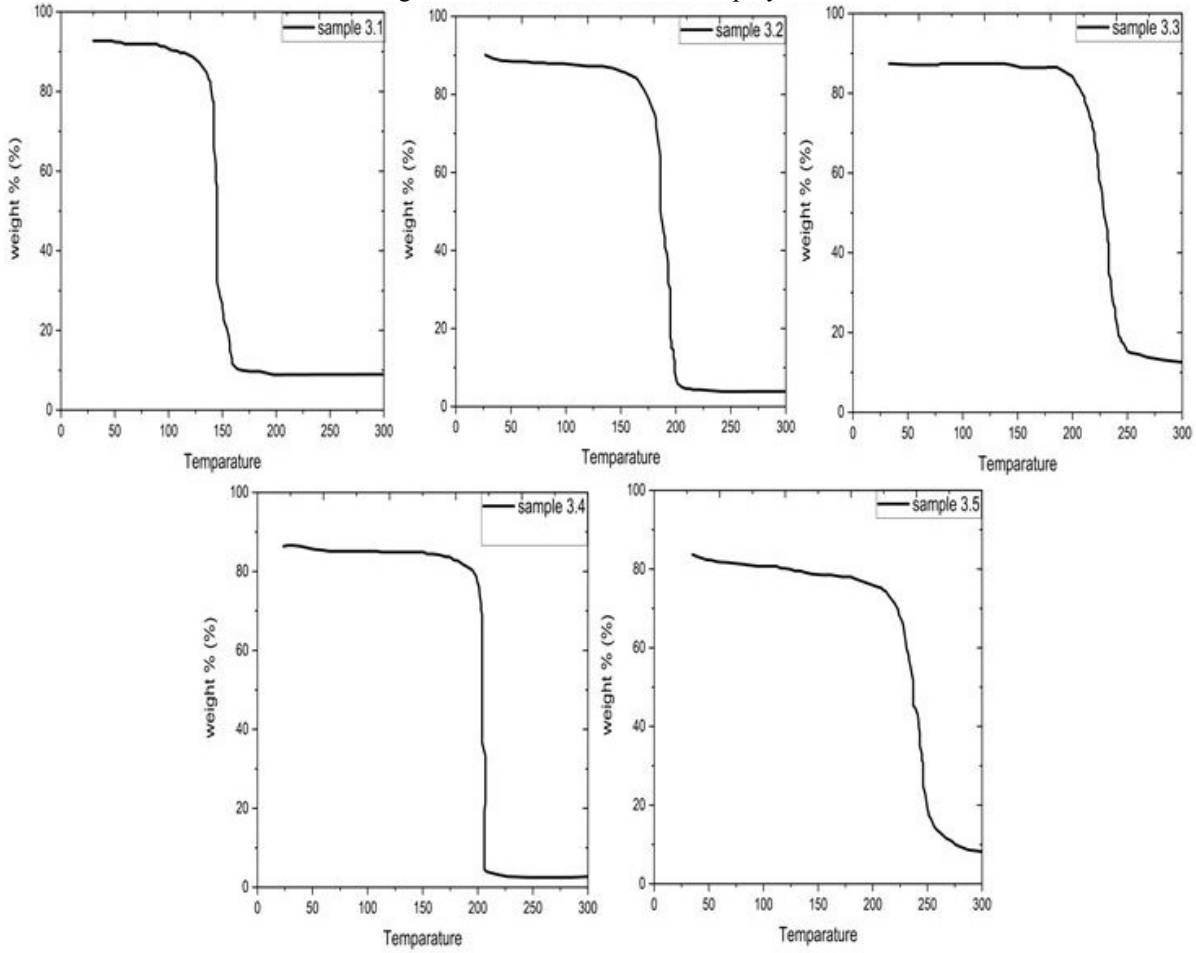

Fig.-2: TGA of Cross-linked Tri-polymers

Essentially, acrylic acid has the glass-transition temperature *(Tg)* is nearly 106°C, but due to cross-linked with DVB, there may be slightly changed in T_g. DSC thermograms designate that T_g of the polymer increases by about 115°C and is independent of the amount of allyl tris buffer. There is the hump in the bend and fix the midpoint in each curve as T_g. The initial stage shows weight loss is between 40°C and 125°C which might be due to the loss of absorbed and bound water. The next stage of mass loss started at 225°C and continued to 275°C, during which weight loss may be due to the degradation of the cross-linker.

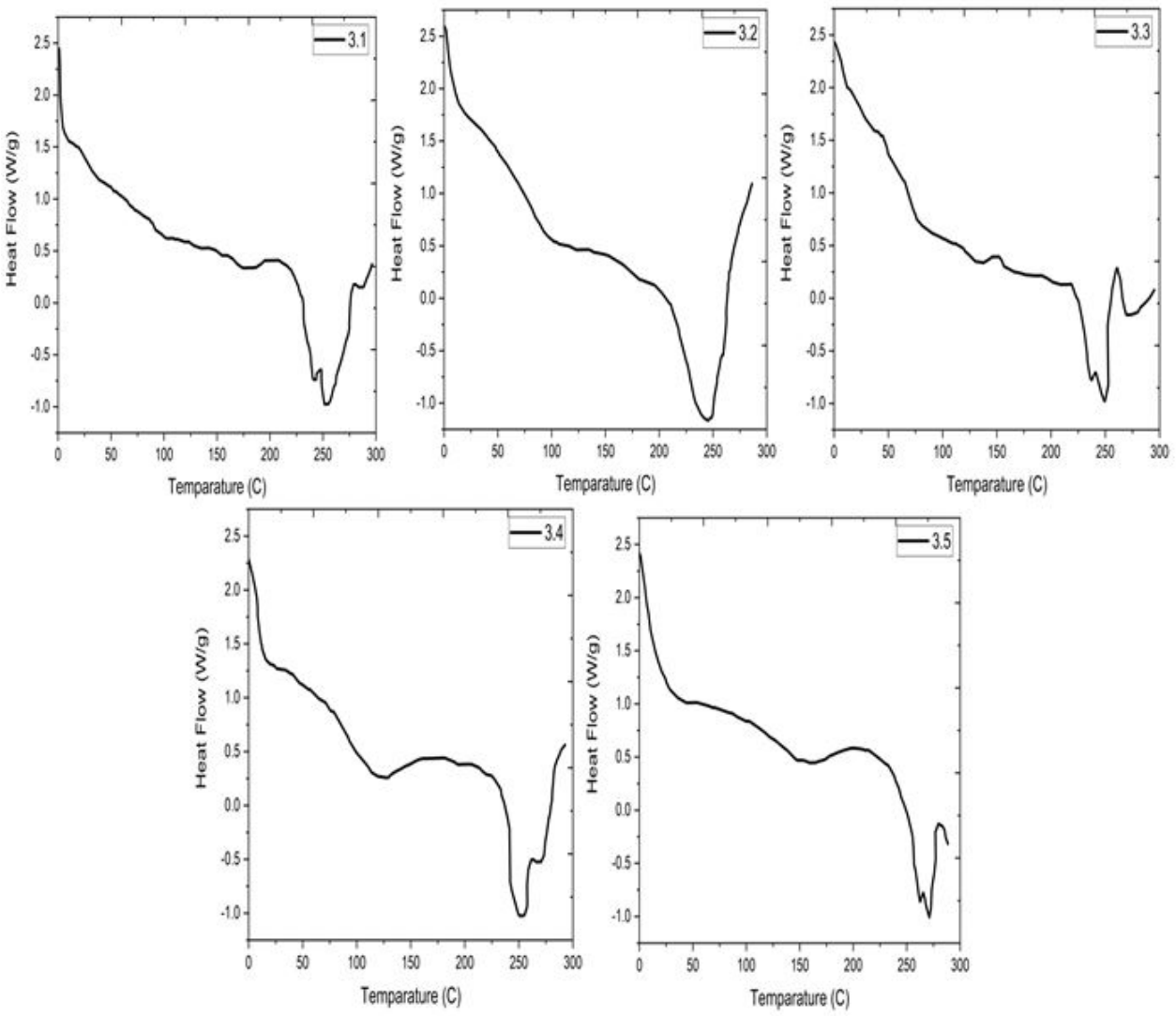

Fig.-3: DSC of Cross-linked Tri-polymers

SEM micrographs of structured cross-linked polymers are investigated and furnished in Fig.-4. The differences are obvious. The super absorbent polymer prepared from allyl tris buffer, acrylic acid and DVB cross-linking (Fig.-4b and c) have a solid amorphous nonporous shell. It can be observed that the fractographs are of a layer-like rough appearance (Fig.-4c), which indicates an adhesive interface. While the further two images of polymer thickeners prepared from DVB, acrylic acids and tris buffer monomers ATB shows different porosity. The highest porosity could be predicted from the monograph of polymer thickening agent cross-linked with ATB, the pores are associated with each other. The porosity can obviously be seen in the inflated view. However, in Fig.-4a aggregation of the porous structure can be seen and seems to be phase-separated.

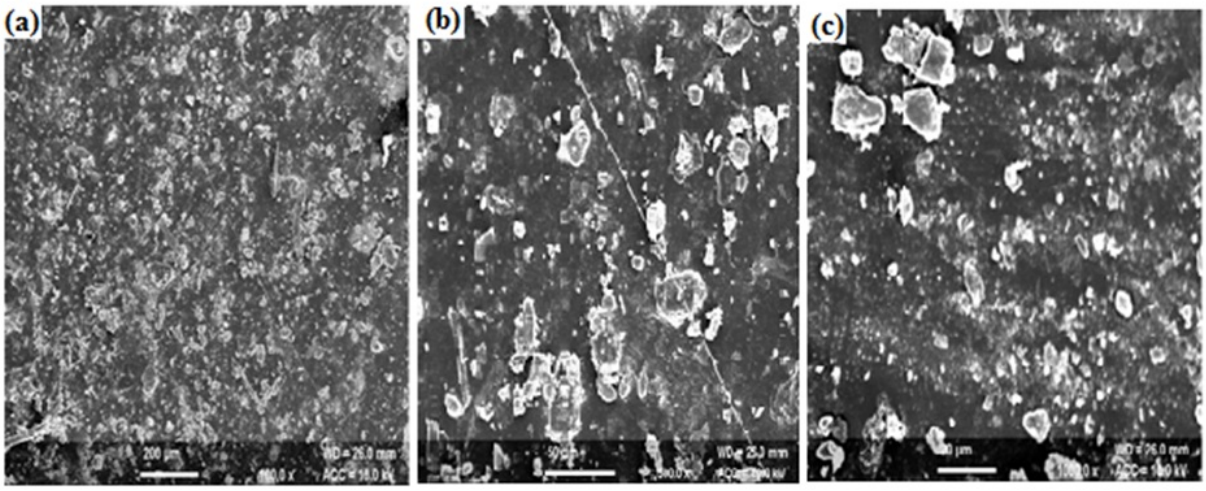

Fig.-4: SEM Micrographs of prepared SAP's shows for the Superabsorbent Polymer Cross-linked with PAA-ATB and Divinyl Benzene with Different Micro Sizes

Weight 0.5 g samples of absorbent polymer and placed into a beaker. The beaker is pre-weighed (W_0). The polymers are dipped in an excess quantity of water for the night to achieve the steadiness of swelling. After swelling, removes the excess volume of water waiting no fluid was drop-offs. The absorbed polymers are weighed (Ws) and the capacity of swelling is calculated by the equation:

$$\text{Swelling capacity} = (\text{swollen/dry}) \times 100\%$$
$$= (W_s / W_0) \times 100\%$$

Table-2: Absorbance Capacity of the Polymer

Weight of Cross-linking Agent (g)	Weight of Sample (g)	Weight of Swelled Polymer (g)	Swelling Capacity %
1.0	0.5	5.782	1057
0.8	0.5	8.012	1502
0.6	0.5	10.108	1922
0.4	0.5	11.614	2223
0.2	0.5	7.750	1450

CONCLUSION

Allyl tris buffer is hydrophilic, acrylic acid is also hydrophilic but DVB is hydrophobic. Since divinyl benzene is far in excess compared to allyl tris buffer, the polymer segments of acrylic acid/DVB form of one phase and ally tris buffer with acrylic acid form the immiscible second phase. By using the presented synthetic method clear rubbery gel polymer product is achieved. The surface morphology and thermal behavior are studied using SEM and TGA techniques, respectively.

The DSC of cross-linked SAP with allyl tris buffer shows a weight loss at 40°C. The super absorbent polymer synthesized from acrylic acid and the cross-linking agent has a solid smooth non-porous surface. Polymer started to degrade at about 150°C and completed nearly 260°C. The cross-linking was also supported by DSC analysis. According to swelling property of SAPs which are absorbs a larger amount of water. SAPs have many applications. Superabsorbent polymers have the capacity to intellect environmental variations, changes of pH, temperature. Hydrophilic networks are reactive to some molecules that can be used as in drug systems, hygienic products like diapers, and in controlled release drugs.[12] Superabsorbent polymers were also employed in various applications, such as absorbent paper products, bandages and surgical pads, wound dressings, and as chemical absorbents. Additionally, they are applicable to food packaging.[1]

The polymers are characterized using SEM patterns and FTIR spectra. The following conclusions are made based on the results and discussion.

i. FTIR spectra exhibit the cross-linking of monomers and cross-linker.
ii. From the swelling analysis, the newly synthesized polymers are absorbing more amount of water content, and they can be, therefore, employed as a super absorbent in various applications.
iii. In the DSC thermograms, weight loss may occur due to the degradation of the cross-linker.
iv. It can be observed that the SEM fractographs are of a layer-like rough appearance solid amorphous non-porous shell.

ACKNOWLEDGMENT

The authors are thankful to the Research Laboratory of Pramukh Swami Science and H D Patel Arts College, Kadi for providing research facilities.

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[RJC-5526/2019]

Medicinal Plants
Vol. 12 (2), June 2020, 236-242

doi : 10.5958/0975-6892.2020.00031.3

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Research Article

Impact of seasonal variation on 'daidzein' accumulation in callus and *in vivo* parts of *Pueraria tuberosa* (Willd.) DC

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Received: April 17, 2020; Accepted: May 05, 2020

ABSTRACT

Daidzein-natural isoflavones found in *Pueraria tuberosa* (Willd.) DC. (Fabaceae) which is a potent medicinal plant. It acts as signal carriers and responds to the pathogenic attacks and reduces pain during menopause, osteoporosis and also antidiabetic in nature. In the present work, *in vitro* callus development from tuber explants during both rainy and summer seasons was conducted. Further, a simple unambiguous and rapid high-performance thin-layer chromatography method was established for quantitative estimation of daidzein in callus and *in vivo* parts to assess the impact of seasonal change on valuable phytochemical accumulation. Maximum callusing (90 %) was obtained on MS medium fortified with a combination of ^{6}N-Benzylamino purine (2 mg/l) and 2,4-dichlorophenoxyacetic acid (2 mg/l) during the summer season from the tuber explant. During the HPTLC method validation, the linearity range obtained was 100-1000 ng/spot with a regression value (r) value of 0.99845. All the parts were found to contain a significant amount of daidzein. The maximum daidzein (2112.567±0.35 ng/g) content was obtained from a young tuber bark followed by callus (171.903±0.33 ng/g) during the summer season compared to rainy season parts and callus. Thus, it can be concluded that *in vitro* callus is an alternative source of daidzein without destroying the natural plant and the developed HPTLC method could be used for quality control analysis and recommended for daidzein quantification for different herbal formulation and drug preparation.

Keywords: Callus, daidzein, HPTLC, isoflavonoids, Indian Kudzu

Abbreviations: BAP = ^{6}N-Benzylamino purine; 2,4 D = 2,4-dichlorophenoxy acetic acid; DZ = Daidzein; Kinetin = 6-furfrylaminopurine; MS = Murashige and Skoog; PGR = Plant Growth Regulator; PT = *Pueraria tuberosa*

INTRODUCTION

Pueraria tuberosa (Willd.) DC (family Fabaceae) is a perennial woody climber and commonly known as Vidarikanda or Indian kudzu and the tuberous roots of this plant are prominently used in the Indian system of Ayurvedic medicine. It is an important medicinal plant for its laxative, emollient, aphrodisiac, diuretic, galactagogue, emetic and cardiotonic properties as described in Ayurveda, (Sadguna and Mustafa, 2012). The plant is a substantial resource as it contains several bioactive compounds and especially its tuber contains many isoflavonoids which are puerarin, daidzein (DZ), genistein genistin, etc. In recent investigations, DZ is known to have antithrombotic, antiallergic, hypolipidemic, antioxidant, hepatoprotective, anti-inflammatory, immunomodulatory, nootropic, anxiolytic, neuroprotective, wound healing and antidiabetic properties (Zhang *et al.,*1997; Vaishnav *et al*., 2006; Ae Park *et al*.,

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2006; Choi and Kim, 2009; Liu *et al*., 2006; Zeng *et al*., 2010; Yang *et al*., 2012; Mukai *et al*., 2012; Choi *et al*., 2014). PT is a multipurpose plant and its tuber is mainly used as a raw vegetable, cooking, decorating and treating many diseases. Though plant tissue culture techniques successfully implicated in PT (Rathore and Shekhawat, 2009) but it could not able to succeed in the availability of its parts for medication or drug production. The *in vitro* cell and tissue cultures considered as the alternative method to produce bioactive compounds from culture rather than depleting the plant in nature. Furthermore, in the medicinal plant, the regional, seasonal and age variations highly influence the extraction of active ingredients (Panda and Kamble, 2016). The constituents and active principles vary quantitatively at different seasons of the year and the majority of plant materials are usually best collected during the season when the herbs are at peak maturity and concentration (Singh, 2008). However, no much emphasis has been given on a comparative account of the number of phytocompounds in *in vivo* and *in vitro* parts separately till date. Also, earlier reports suggested that the environmental factors and seasons do have an influence on the cellular accumulation of such important phytocompounds in many medicinal plants (Jayanthy *et al*., 2013; Wojciak-Kosior *et al*., 2016). Moreover, seasonal variations make the plants more vulnerable to different temperature regimes and show an immense influence in their phytochemical constituents, with volatile compounds being the most deteriorated (Usano-Alemany *et al*., 2014). Thus, the deduction of the seasonal consequences on plant phytochemical constituents provides an idea on the schedule (time/season) of the harvest of individual plant species that have an ideal amount of active ingredients (Kale, 2010). Nowadays, the HPTLC technique is being widely used for analytical quantification of these chemical markers through chromatographic separation on a silica gel layer, along with densitometric quantification of the separated compounds. The obtained results from the HPTLC analysis is a reliable, quick, accurate, and proportionately cost-effective method of quantification (Misra *et al*., 2017). Previously, some reports on the quantification of important bioactive compounds from *in vivo* and *in vitro* plant parts were observed (Parale *et al*., 2010; Pandey *et al*., 2016; Panigrahi *et al*., 2017). The present investigation was aimed to find out the impact of seasons in terms of summer and rainy on in *vitro* tissue development and differential accumulation of DZ in it and HPTLC quantification of DZ both from *callus* and *in vivo* parts of PT.

MATERIALS AND METHODS

Plant material

The multiple plant parts like stem, leaf and root tubers of different sizes of *Pueraria tuberosa* (Willd.) DC. were collected by digging nearby soil up to a depth of one to one and a half meters of the bushy hedges and tall tree on the support of which *Pueraria* climber available in the forest region of Mota limtawada, Taluka-Rajpipla, District Narmada, Gujarat, India during the onset of the rainy season (July-August, 2017) and summer season (February-March, 2018). The materials were identified and authenticated by the Botany Section of the Department of Life Sciences, Hemchandracharya North Gujarat University, Patan, Gujarat, India.

Callus development

The fresh leaf, stem, young root tubers (small tubers with 2 to 3 cm diameter and 4 to 6 cm length and 100 to 200 g fresh weight) and old tubers (large tubers with 5 to 8 cm diameter and 10 to 12 cm length and 500 to 1000 g fresh weight) were collected during both the seasons and thoroughly washed to remove soil darts followed by 1% streptomycin treatment about 15 min for sterilization. Later on, these tubers immersed in 70% ethanol for1 min followed by surface sterilization in 0.5% mercuric chloride solution up to 15 min. Further, these tubers cleaned six times in sterile distilled water. The sterilized root tubers were chopped into small pieces and inoculated aseptically into MS medium (pH= 5.7 before addition of gelling agent) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar along with varied concentrations of kinetin (0.5, 1.0 mg/l), BAP (6-Benzylamino purine) (0.5, 1.0, 2.0 mg/l) and 2,4-D (2,4-Dichlorophenoxy acetic acid) (0.5, 1.0 mg/l) and combinations of BAP + 2,4-D (0.5+0.5, 1.0+1.0, 2.0+2.0 mg/l). The culture was incubated at 26±2°C under a 16 h illumination at a light intensity of 3000 lux fitted with cool white fluorescent tubes and periodic data were recorded for callus development. The results were furnished as the mean value with SD (standard deviation). The data were interpreted by one-way analysis of variance (ANOVA) followed by Duncan multiple comparisons (p <0.05) based on SPSS software (SPSS for Windows 20.0, SPSS Inc., USA).

HPTLC method validation and quantification

Preparation of standard solution

A stock solution of DZ was prepared by dissolving 1 mg of accurately weighed DZ in 1ml methanol and the stock solution was diluted to the obtained concentration at 100 ng/1µl.

Extraction procedure

In vivo leaves, stem, young and old tuber barks and pulp separately and *in vitro* developed callus (4 weeks) of both seasons i.e. rainy and summer were collected separately. All the collected samples were shade dried, coarsely powdered by using a mechanical grinder and stored in airtight containers. 1 g powdered samples were extracted using 10 ml methanol and kept on a shaker for 24 h at room temperature and used further for HPTLC analysis.

HPTLC instrumentation and validation

The pre-coated with silica gel 60 F254 plates 20 x 10 cm aluminum plates were chosen where the standard and sample solutions were spotted as 8 mm wide bands with TLC applicator Linomat V supported with N_2 flow (CAMAG, Switzerland). The mobile phase toluene: formic acetic acid 99:01 (v/v) was used for the development of the above plates under CAMAG twin trough glass chamber for 20 min. The developed height was allowed up to 80 mm under 25±2°C and 50 ± 2% relative humidity. The TLC plate was scanned at 254 nm light. The HPTLC analysis was operated following the International Council of Harmony guidelines (ICH, 2006). The method was validated by operating linearity, specificity, reproducibility, and accuracy (recovery) using standard and samples.

Quantification of DZ from callus and in vivo samples

The developed and validated HPTLC method was used for quantification of DZ from callus and multiple *in vivo* plant parts obtained during both rainy as well as summer season.

RESULTS AND DISCUSSION

Callus development

The fortification of PGRs alone or in combination produced callus in PT tuber explant. The combinations of BAP and 2,4-D took 13 days for initiation of callus both with 1 mg/l plus 1 mg/l and 2 mg/l plus 2 mg/l during the summer season (Table 1). The maximum days for callus induction were obtained by the application of 0.5 mg/l 2,4-D in both the season 25 and 21 days rainy and summer season respectively. The individual BAP and kinetin did show response in callus

Table 1: Effect of different plant growth regulator (PGR) combinations on callus development of PT

Plant growth regulators types	PGRs (mg/l)	Rainy season			Summer season		
		Response initiation (days)	% Growth frequency	Callus characteristics	Response initiation (days)	% Growth frequency	Callus characteristics
Control	-	-	-	-		-	-
2, 4 –D	0.5	25	13.33±3.33e	Creamish, soft	21	20.00±5.78ef	Whitish brown, friable
	1.0	22	26.66±6.67de	Creamish, soft	21	33.33±8.82cdef	Whitish brown, friable
BAP	0.5	19	46.66±8.82abc	Brownish, compact	16	23.33±3.33def	Greenish, compact
	1.0	19	33.33±3.33cd	Brownish, compact	16	43.33±3.33bcd	Greenish, compact
	2.0	18	50.00±5.78ab	Brownish, compact	16	56.66±6.67b	Brownish, compact
Kin.	0.5	21	16.66±3.33e	Pale brownish, soft	19	16.66±3.33f	Yellowish, compact
	1.0	21	20.00±5.78de	Pale brownish, soft	19	40.00±5.78bcde	Yellowish, compact
BAP+2,4-D	0.5 + 0.5	20	43.33±3.33bc	Brownish, compact	18	26.66±12.03cdef	Greenish, hard
	1.0+1.0	20	56.66±3.33ab	Brownish, compact	13	46.66±8.82bc	Greenish, hard
	2.0 + 2.0	20	60.00±8.82a	Brownish, compact	13	90.00±5.78a	Greenish, hard

Note: Values represent as mean± SD. Means followed by the same letter within columns are not significantly different ($P < 0.05$) using Duncan's multiple range test

induction (19-21 days). However, the different combinations and concentrations of cytokinin and auxin showed a significant (P<0.05) effect on callus development from tuber explants. During the rainy season, maximum callus development was obtained in MS medium with 2.0 mg/l BAP + 2.0 mg/l 2,4-D as the best hormonal combination with 60.00±8.82% growth frequency. The rainy season callus was initially creamish in color but later on, it turned into brownish color but growth was meager compared to the summer season (Figure 1A). However, during the summer season huge greenish colored, hard mass of callus started to develop in MS + 2.0 mg/l BAP + 2.0 mg/l 2,4-D each with a high growth frequency of 90±5.78% (Figure 1B). As the concentration of PGRs increased, the percentage growth frequency increased during both the seasons up to a certain limit. It was noted that the 2,4-D plays a prominent role in inducing greenish callus from tuber explants. The similar types of callus production obtained by Sadguna and Mustufa (2012) when PT leaves were taken as explant and grown in MS medium fortified with 2,4-D and BAP. Moreover, the results obtained from the present study are in accordance with Zafar and Humayun (2012) in *Clitoria ternatea* and Verma *et al*. (2012) in *Catharanthus roseus*. They reported that the enhanced callus development was obtained on MS medium fortified with lower doses of 2,4-D+BA. Thus, the callus obtained from these combinations of 2,4-D and BAP (2 mg/l + 2 mg/l) of both seasons was collected for HPTLC analysis.

HPTLC fingerprint analysis

The preparatory HPTLC fingerprint analysis displayed that toluene: ethyl acetate: Acetone: formic acid (10:2:1:0.5 v/v) as a mobile phase for the development of the plate with an R_f value of 0.32 for DZ and well-resolved bands were obtained both for callus and *in vivo* parts of both the seasons (Figure 2). Good separation of various flavonoids bands was achieved at 254 nm and the identity of the bands of DZ in the sample was confirmed by comparing their R_f values with reference standards. Figure 2 represents a typical HPTLC plate showing a fingerprint comparison of standard DZ with test samples.

Method validation

The standard curve for the DZ was obtained by performing the linearity curve of DZ that was obtained using working solutions containing 100–1000 ng of DZ that was spotted on the TLC aluminum sheet. The regression equation obtained for the calibration curve was Y=7005.043+1140.108*X. The correlation coefficient for a calibration curve was found to be 0.99551 with a 1.49% standard deviation (Figure 3). The results of linear regression were summarized in Table 2. In order to evaluate the limit of detection (LOD) and limit of quantification (LOQ), a calibration curve was used. The LOD

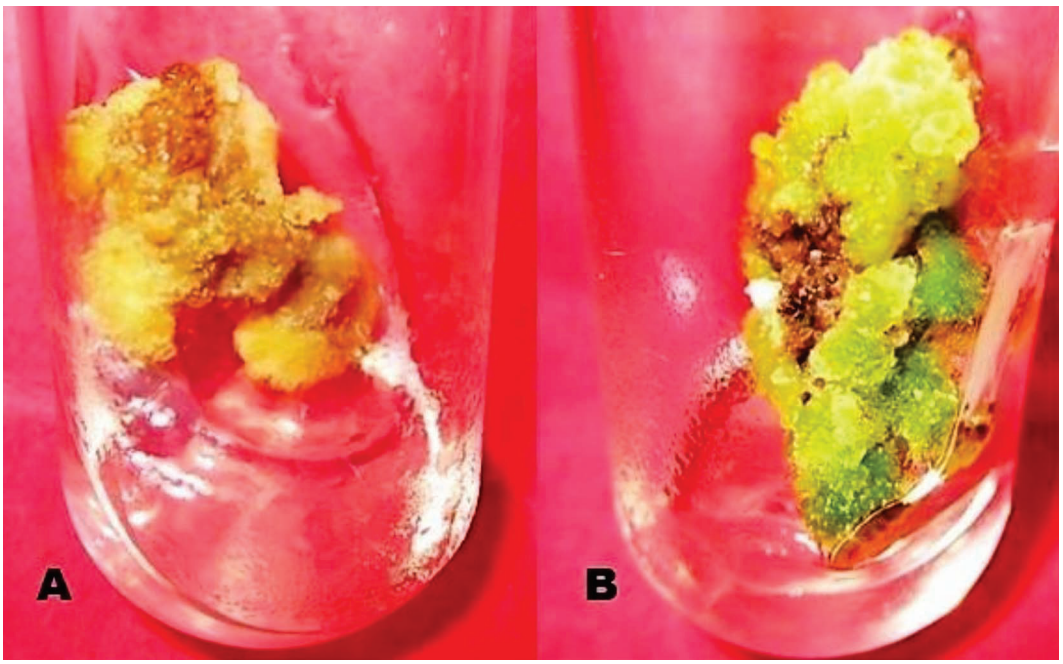

Figure 1: Callus development of *Pueraria tuberosa* during A) rainy and B) summer season after 6 weeks from tuber explants

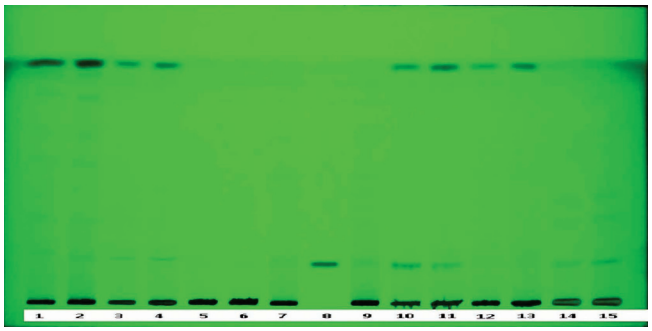

Figure 2: HPTLC finger printing of *in vivo* parts and callus of PT during rainy and summer season with the marker DZ. (1-7 – Rainy Season): 1- callus, 2- old tuber bark, 3- old tuber pulp, 4- young tuber bark, 5- young tuber pulp, 6- stem, 7- leaf, 8- std. Daidzein, (9-15- Summer Season): 9- leaf, 10- leaf, 11- stem, 12- young tuber pulp, 13- young tuber bark, 14- old tuber pulp, 15- callus

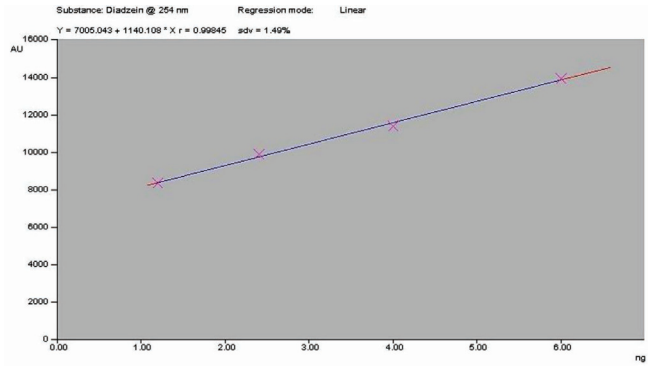

Figure 3: Calibration curve of DZ

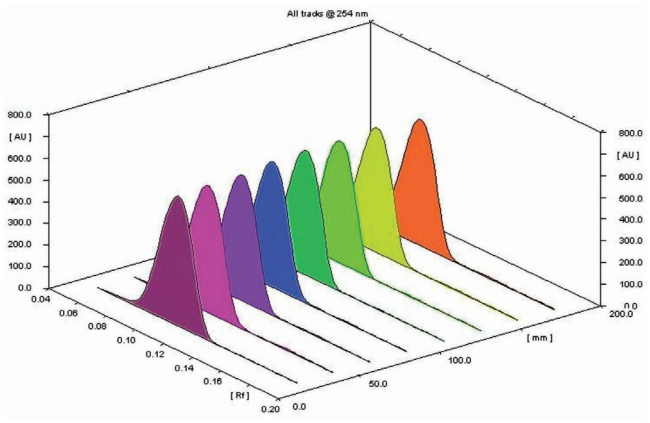

Figure 4: 3-D graph for Reproducibility at 254 nm

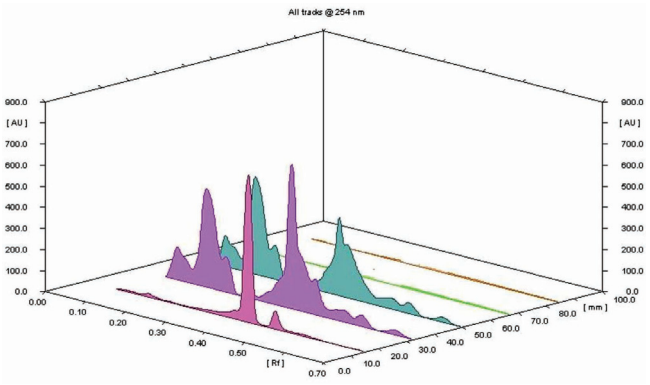

Figure 5: 3-D graph of Specificity DZ at 254 nm

value and LOQ were found to be 356.8 ng and 1081.2ng, respectively. The specificity was performed to determine the purity of samples, standards, diluents and the mobile phase (Figure 4). The specificity was observed to be particular as indicated by the prerequisites. The intra-day and inter-day precision were found to be at 2.21% and 1.19%, respectively. Reproducibility results showed a 3-D graph with uniform humps and spectral scanning demonstrated complete superimposed peak of DZ devoid of any other peaks (Figure 5). Percentage recovery was measured at three levels (80, 100 and 120%) on the plate. The accuracy of the method was found to be at 72.04%. Thus, R_f data confirm the system suitability and accuracy of the instrument through method validation.

Quantification of daidzein from *in vivo* and *in vitro* tissues

The quantities of DZ obtained from callus and various *in vivo* parts were expressed as ng/g of dry weight (Table 3). The accumulation of DZ was less in samples of the rainy season. Among *in vivo* parts, higher DZ accumulation was recorded in small tuber bark of both the seasons but the maximum amount (2112.56±0.35 ng/g) was obtained in the summer season followed by leaf samples (1231.83±0.44 ng/g). Whereas among callus samples, the DZ accumulation was also recorded, but the maximum amount of DZ (171.90±0.33 ng/g) was recorded in callus developed during the summer season compared to the rainy season callus (102.83±0.45 ng/g). The increased callus proliferation during the summer season may help in higher production of DZ in it and this may be due to heat stress-mediated *in vitro* synthesis of secondary metabolites in the plant. Similarly, Vaishnav *et al*. (2006) reported the presence of DZ in various *in vivo* plant organs viz. leaf, stem, root tuber and *in vitro* developed callus of PT through HPLC studies. Likewise, Gololo *et al*.

Table 2: Method validation parameters for the quantification of DZ

Parameters	Observation
Linearity range (ng/spot)	100-1000
Correlation coefficient (r)	0.99845
Regression equation	Y=7005.043+1140.108*X
Sdv	1.49 %
LOD (ng/spot) (Limit of detection)	356.8
LOQ (ng/spot) (Limit of Quantification)	1081.2
Intra assay Precision (RSD [%] n= 6) on the same Day	2.21%
Intermediate Precision (RSD [%] n= 6) after three Day	1.98%

Table 3: HPTLC quantified DZ (ng/g) in different *in vivo* parts and callus of PT during rainy and summer seasons (values are mean ± SD of 3 replications)

Sample name	Amount (ng/g)	
	Rainy season (ng)	Summer season (ng)
Leaf	668.023±0.30	1231.833±0.44
Stem	1.783±0.09	7.45±0.15
Young tuber pulp	9.204±0.05	19.403±0.66
Young tuber bark	921.94±0.31	2112.567±0.35
Old tuber pulp	6.273±0.06	13.10±0.30
Old tuber bark	24.938±0.47	51.363±0.46
Callus	102.833±0.45	171.903±0.33

(2016) reported that, the maximum amount of flavonoids and phenols obtained during summer in plants like *Barleria dinteri, Grewia flava* and *Jatropha lagarinthoides*. The results of higher DZ accumulation in small tuber bark/ skin part rather than pulp and in any other parts during summer seasons indicated that age or stage of development of any organ or plant part also has a great impact on the quantitative accumulation of any bioactive compound in it.

The *in vitro* system of culture is reckoned as an important technique for the valuable productions of phytochemicals and with true medicinal principles (Gantait and Panigrahi, 2018). The PGRs applied in in vitro studies was enhanced some of the metabolic features along with phytochemical accumulation in *in vitro* tissues unlike the *in vivo* equivalent parts (Pandey *et al*., 2016). The progress in tissue culture and cell culture techniques can lead to secondary metabolite productions on a large scale (Debnath *et al*., 2006; Panigrahi *et al*., 2017). At certain times the in vitro tissue yielded more secondary metabolites than its counterparts (Karuppusamy, 2009; Panigrahi *et al*., 2017). These environmental factors are already reported to have an effect on the quantities of phytochemicals in certain plants (Usano-Alemany *et al*., 2014). The increased levels of total alkaloids and polyphenols in leaves during summer seasons were also recorded by Sahoo *et al.* (2012) in *Barleria prionitis, Boerhavia diffusa, Citrillus colocynthis,* and *Grewia tenax.* The current study also showed increased amounts of flavonoids specially DZ accumulation during warmer seasons in all *in vivo* parts and callus tissues and they were found potential source for production of DZ but for this one has to be very careful for explants harvesting and inoculation time. For *Pueraria tuberosa* (Willd.) DC., summer season was found better for *in vitro* tissue development as well as for DZ accumulation.

CONCLUSION

The impact of seasonal variation on the secondary metabolites is considered to be an important aspect to identify the perfect season with high amounts of DZ accumulation in *Pueraria tuberosa*. The *in vitro* callus formation from tuber explant was profused during summer season compared to rainy may be due to presence of increased endogenous hormones and bioactive substances in tuber during summer. The work also concluded that important isoflavones such as DZ could be accumulated with high amounts during summer season in all the *in vivo* parts and *in vitro* callus reflects that the heat stress induces accumulation of different bioactive principles possess in plant. Therefore, one plant species may be harvested/collected for phytochemical point of view during allied seasons and at particular age of it resultant in enhanced accumulation of bioactive compounds. Thus, the right time and right season of harvesting are recommended for DZ and other similar isoflavonoid collection from PT. The present study would be useful for herbal formulation and bring down the cost of DZ by the simultaneous complementation of callus and *in vivo* parts and it will also restrict the overexploitation of this precious plant in nature.

Conflict of interest: None

ACKNOWLEDGMENT

Authors are thankful to the Gujarat State Biotechnology Mission (GSBTM, Gandhinagar, Gujarat India) for funding this research as a major project.

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