RESEARCH ARTICLE

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Exploration of Acemannan as a Phytomarker for *Aloe barbadensis* Gel and its Estimation in Different Marketed Cosmeceutical Formulations

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Abstracts: *Aloe barbadensis* (*Aloe vera*) is a well-known plant belonging to family liliaceae with rich source of anthraquinone glycosides and polysaccharides. *Aloe vera* is used extensively in the cosmetic and healthcare industries. However estimation of active constituents of aloe in any of the marketed product is not reported yet because of lack of availability of suitable phyto-marker. Hence, in the present investigation attempts have been made to identify phyto-marker from the aloe juice and exploration of identified marker as tool for estimation of aloe in marketed cosmeceutical products. From the published research report it has been observed that activity of aloe vera is due to the presence of several phytonutrients and polysaccharides like acemannan. Acemannan, specifically in aloe vera has been identified but not explored extensively as a marker for detection of aloe vera gel in cosmeceutical formulations. Further, the estimation technique based on spectroscopy i.e. nuclear magnetic resonance (NMR) / fourier transform infrared (FT-IR) and chromatographic i.e. gel chromatography/size exclusion high performance liquid chromatography (SE-HPLC) has been reported for estimation of acemannan, but these techniques require sophisticated instruments and are expensive as well as laborious which make them unsuitable for routine quality control checks for acemannan in the formulations. In view of the reported constraints, the present work focuses on a development and validation very simple and sensitive colorimetric assay method for estimation of acemannan in an *aloe vera* containing products with extended calibration curve for improved detection. In present exploration, estimation sensitivity of acemannan was enhanced by derivatization using congo red dye which make stable congo red-aloe polysaccharide chromogenic complex. Three formulations namely Lab scale developed cream, Marketed Formulation 1 and Marketed Formulation 2 were studied with acemannan as a phytomarker for aloe vera. The developed spectrophotometric method accurately measured acemannan in the sample, which was implicated by stable red color in the presence of Congo red. Overall proposed spectrophotometric technique for determination of aloe polysaccharide in different formulations was found to be robust, accurate, sensitive and precise for routine quality analysis of aloe vera in cosmeceuticals formulation.

INTRODUCTION

Aloe barbadensis Miller (*Aloe vera*, liliaceae) is commonly referred as first aid plant or burn plant and extensively used in cosmeceuticals products [1, 2]. *Aloe vera* leaf contains two principal components, the yellow latex (exudates) from pericyclic cells and the clear inner leaf gel from parenchyma cells, which is responsible for its physiological and pharmacological effects. *Aloe vera* is largely used for cosmeceuticals industry because it stimulates the immune system and possesses excellent skin moisturizer, antibiotic, anti-inflammatory as well as antiseptic properties [3]. Many leading cosmetic companies have marketed skin and hair care products containing *aloe vera* extracts. *Aloe vera* gel is employed as an active ingredient in variety of skin lotion, sun block creams and cosmetics due to its skin moisturizer, cleanser, bleach and a rejuvenator potential [4]. *Aloe vera* also explored potentially in the fields of baby products like baby lotions and wipes [5-6]. Researchers have also investigated and reported anti-tumor, antiviral, anti-rheumatoid and anti-diabetic properties of aloe vera [7-9].

The major active constituent isolated and identified from the aloe juice is hydroxyl-anthracene derivative known as barbaloin (Figure 1; aloin). It also contains hydroxyloin of about 3% w/w. Barbaloin is a mixture of aloin A (10S; Figure 1(a)) and aloin B (10R; Figure 1(b)). Aloin A and aloin B are interconvertible [10]. But aloin is not employed as the marker for estimation of aloe vera gel for the standard of cosmetic products of aloe because it is present in yellow exudates and cosmetics products mostly contains the clear mucilaginous gel.

Aloe gel is also contains glycoproteins, polysaccharides, and enzymes, which are used essentially in the treatment of various skin conditions such as burns, abrasions, bruises, cuts, psoriasis, and herpes simplex [3]. Acemannan (Figure 2) was found as principle polysaccharides present in the aloe gel. Acemanann is a sensitive polysaccharide that undergoes rapid degradation if not stabilized against aloe endogenous hydrolyzing enzymes and also may degrade under high temperature condition. Because of the large number of marketed cosmetic products containing either aloe gel or its constituents, it is necessary to develop simple, rapid and accurate analytical technique to estimate the acemannan in a formulation to ascertain the quality of finished products.

In order to identify the aloe gel in the cosmeceuticals acemannan was explored as a potential phytomarker. Lab Scale cream containing different concentration of acemannan was prepared and evaluated using developed estimation technique.

MATERIALS AND METHODS

Instrument and Materials

Instrument used were UV 2450 Pc series double beam UV/Visible Spectrophotometer (Schimadzu), Citizen CX 220 analytical balance and probe as well as bath type sonicator (Trans-o-Sonic). *Aloe vera* leaves were

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Table 1: Composition Lab Scale Developed Cream Containing *Aloe vera* Gel

S. No.	Ingredient	Quantity Used
1	Carbopol 934	0.15 g
2	Glycerin	2ml
3	Cetyl alcohol	4g
4	Stearic acid	8g
5	Mineral oil	5ml
6	PEG 200	3.5ml
7	Aloe vera gel	10 gm
8	Methyl paraben	0.1g
9	Water	30ml
10	Flavor	q.s.

Table 2: Spectrophotometric Method Validation for Quantitative Colorimetric Analysis of Acemannan

Acemannan Stock Solution Conc. (µg/ml)	Stock Solution Volume (ml)	Congo Red Solution (ml) (0.01 % w/v)	Concentration of Acemannan in Complex (µg/ml)	Absorbance	% Bias	Accuracy
100	0.4	4	9.09	0.05	0.00	100.00
200	0.4	4	18.18	0.088	-11.99	94.45
500	0.4	4	45.45	0.23	-7.99	115.57
800	0.4	4	72.73	0.38	3.26	101.44
1000	0.4	4	90.91	0.485	5.43	93.27
1200	0.4	4	109.09	0.612	5.16	102.23
1500	0.4	4	136.36	0.722	-0.75	97.60
1800	0.4	4	163.64	0.802	-7.44	95.77
2000	0.4	4	181.82	0.88	-8.59	93.48

Table 3(a): Results of Repeatability Studies

Concentration of Acemannan in Complex (µg/ml)	Absorbance 1	Absorbance 2	Absorbance 3	Absorbance Mean	SD	%RSD
9.09	0.045	0.050	0.043	0.046	0.004	0.838
18.18	0.085	0.088	0.079	0.084	0.005	1.657
45.45	0.260	0.230	0.220	0.237	0.021	0.796
72.73	0.422	0.380	0.418	0.407	0.023	1.700
90.91	0.485	0.485	0.500	0.490	0.009	1.767
109.09	0.595	0.612	0.621	0.609	0.013	1.167
136.36	0.710	0.722	0.700	0.711	0.011	1.550
163.64	0.816	0.802	0.810	0.809	0.007	0.868
181.82	0.885	0.880	0.877	0.881	0.004	0.459

Table 3(b): Results of Intraday Precision

Concentration of Acemannan in Complex (µg/ml)	Absorbance	Mean Absorbance	SD	% RSD
9.09	0.045 0.045 0.046	0.045	0.001	1.274
90.91	0.485 0.485 0.491	0.487	0.003	0.593
136.36	0.710 0.722 0.720	0.717	0.006	0.896

collected from the medicinal garden, Institute of Pharmacy, Nirma University, in the month of January 2010 and gel of the leaves was isolated and used for analysis. Congo red dye, ethyl alcohol, trifluoro acetic acid were purchased from S.D. Fine chemicals. All other chemicals and reagents used were of analytical grade. *Aloe vera* based products (cream) were purchased from market.

Plant Material

Fresh *aloe vera* leaves were collected from the medicinal garden, Institute of Pharmacy, Nirma University, in the month of January 2010. Whole leaves were washed with distilled water to remove dirt from the surface. The spikes along their margins were removed before slicing the leaf. The epidermis was carefully separated from the parenchyma using a scalpel. The filets were extensively

Table 3(c): Results of Interday Precision

Concentration of Acemannan in Complex (µg/ml)	Absorbance	Mean Absorbance	SD	%RSD
9.09	0.056 0.055 0.054	0.055	0.001	1.818
90.91	0.503 0.506 0.495	0.501	0.006	1.134
136.36	0.723 0.728 0.733	0.728	0.005	0.687

SD = Standard deviation
RSD = Relative standard deviation

Table 4: Method Specificity

Specificity	Sample	Water	Congo-red Dye 0.01%	Acemannan (1mg/ml)	Guargum (2mg/ml)	Tragacanth (2mg/ml)	Absorption 540 nm
Only Sample	Blank	0.8ml	4ml	---	---	---	Reference
	Sample 1	---	4ml	0.4ml	0.4ml	---	0.094
	Sample 2	---	4ml	0.4ml	---	0.4ml	0.098
	Blank	0.8ml	4ml	NA	NA	NA	Reference
	Sample	---	4ml	0.4ml	---	---	0.094

Table 5: Validation Parameters

S. No.	Parameters	Results
1	Absorption Maxima (λ_{max})	540 nm
2	Linearity range	9.09µg/ml-181.82 µg/ml
3	Regression Equation	y = 0.0047x + 0.0027
4	Correlation coefficient (R^2)	0.9942
5	Intercept	0.0027
6	Slope	0.0047
7	Precision Interday %RSD (n=3) Intraday %RSD (n=3)	2%<
8	Limit of Detection (LOD)	2.55 µg/ml
9	Limit of Quantification (LOQ)	8.51 µg/ml
10	Recovery	90 -95%
11	Specificity	A 90.91 µg/ml solution of acemannan-congo red complex solution at UV detection of 540 nm will show an absorbance value of 0.489.

Table 6: Analysis of Different Formulations

Formulation	Label claim(g) of Aloe Vera extract	Amt of APS Should be Present(µg)	Amt of APS estimated(µg)(n=3)	Assay (% ±SD) As compare to labeled claim
Marketed Formulation 1	112.5 112.5 112.5	168.75 168.75 168.75	68 70 75	42.07±3.60
Marketed Formulation 2	7.5 7.5 7.5	11.25 11.25 11.25	10.33 10.15 10.5	92.84±0.175
Lab scale developed cream	10 10 10	15 15 15	14.33 14.50 14.43	96.10±0.08

washed with distilled water to remove the exudate from the slices. Fresh aloe filets were stored no longer than 1 h at 1°C prior to dehydration.

Extraction of the Gel from Leaves of *Aloe barbadensis*

Fresh *aloe vera* leaves (100gm) were used as the raw material. Whole leaves were washed with distilled water to remove dirt from the surface. The longitudinal cuts were made to the leaves surface and kept over for 5 min to drain out the yellow exudates. Furthermore, the mucilaginous parenchyma tissue of the aloe leaf was carefully separated from the skin. Fresh aloe filets were homogenized in a blender, and were filtered through eight layers of cheesecloth [11].

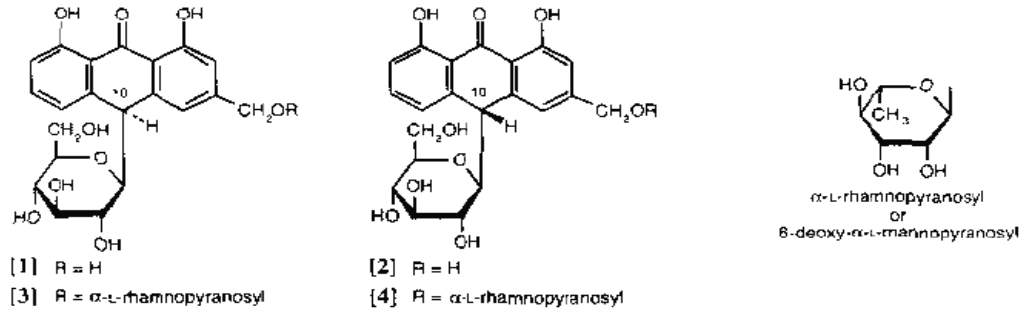


Figure 1: Barbaloin mixture

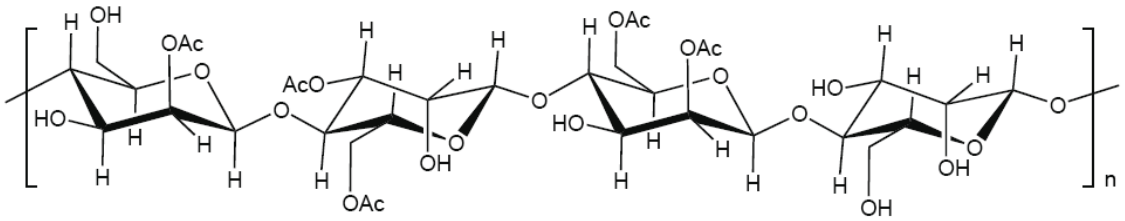


Figure 2: Structure of polydispersed b-1, 4-linked mannan substituted with O-acetyl groups (acetylated mannan; aloe polysaccharide/acemannan)

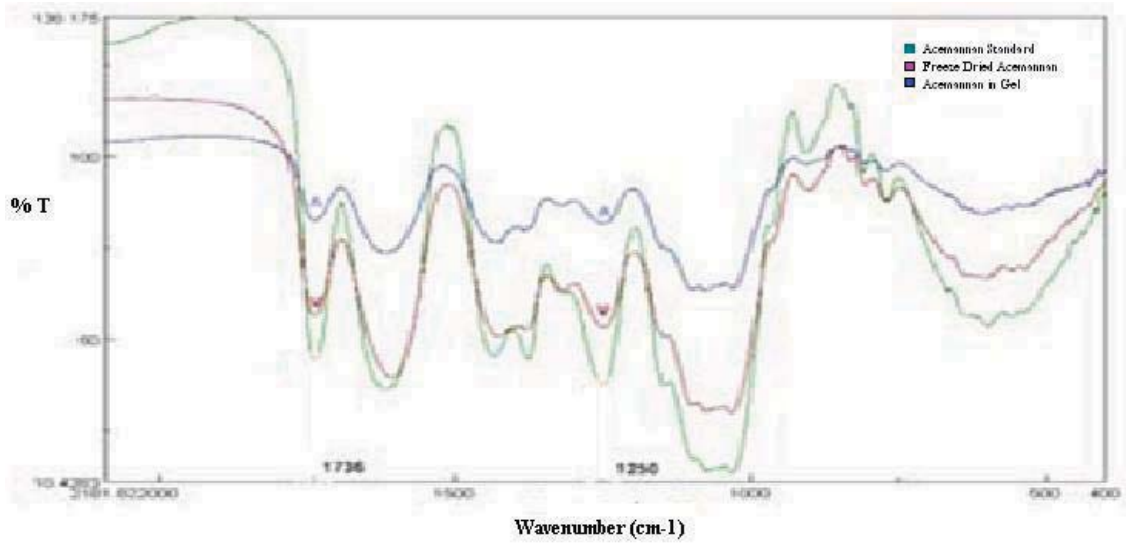


Figure 3: Overlay FT-IR spectra of acemannan standard, freeze dried acemannan (Isolated) and acemannan in gel

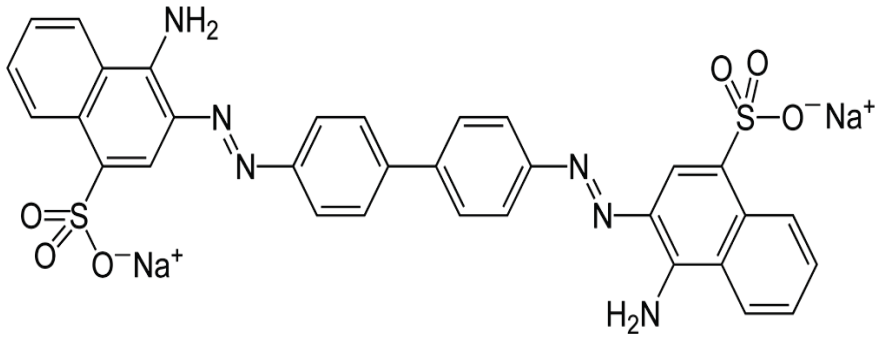
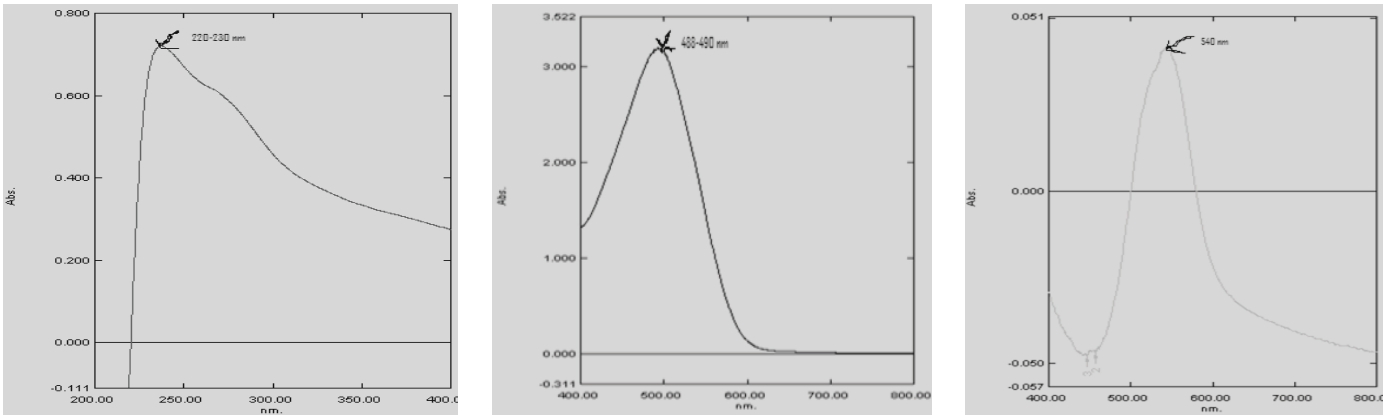


Figure 4: Congo red dye

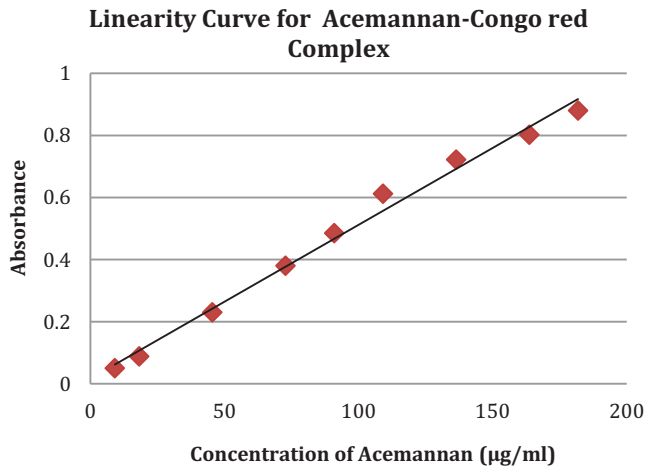
Isolation of Acemannan from the *Aloe barbadensis gel*

20 gm of aloe vera gel was collected as per the procedure describe above and used after filtration [3]. The pH of the filtrate was adjusted to 3.2 with 6mol/L hydrochloric acid. Absolute alcohol was added to the gel to achieve gel to alcohol ratio at 1:4. The mixture was stirred for 30 min, and then allowed to stand for 4 hrs. Then after the mixture was centrifuged at 4000 rpm for 10 min to collect the



(a) (b) (c)

Figure 5: Absorption maxima of (a) acemannan, (b) congo-red dye (0.01 % w/v) and (c) acemannan-congo red (0.01 % w/v) complex



Regression Equation Y = 0.0050X + 0.017
Regression co-efficient R^2 = 0.9942

Figure 6: Calibration curve for acemannan complexed with congo-red

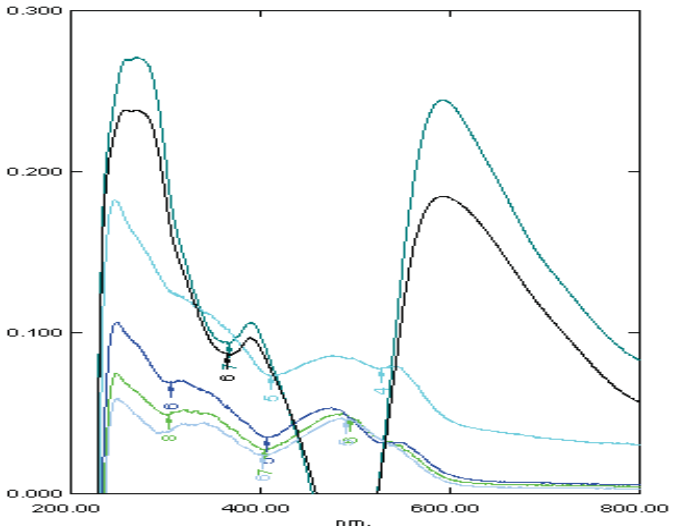


Figure 7: Overlay spectrum of calibration curve of acemannan

precipitated mass and washed with 80% alcohol followed by 3 cycles of centrifugation and distilled water washings to remove the unwanted materials. The crude product of aloe polysaccharide obtained as precipitate was subjected to lyophilization for 24 hrs (INSTRUMENT NAME) and stored at 4°C till further use. The lyophilized product of aloe polysaccharide was dissolved in water (50 ml) during further processing and filtered through whatman filter paper to remove the insoluble residue. The filtrate was de-proteinized by addition of tri-chloro-acetic acid (0.01%) and the process was repeated thrice, the de-protenized solution was collected and allow standing for 2 hrs. The collected de-protenized solution was subjected to continuous dialysis against redistilled water for 48 hrs. with gradual addition of absolute alcohol into the dialysis media to attain 80% alcohol concentration, which induced complete precipitation of aloe polysaccharide from the solution. Subsequently, the precipitated polysaccharide was collected and purified by repetitive centrifugation followed by washing cycle with 80% alcohol. The purified precipitated mass of polysaccharides was dehydrated with anhydrous diethyl ether and subjected to lyophilization to obtain the referential standard product of acemannan [11, 12].

Identification of Isolated Acemannan

FTIR spectrum of isolated acemannan and standard acemannan were obtained by the method of KBr cell on a FTIR spectrometer (Jasco 6100, Japan) with resolution of 2 cm^{-1}. A total of 16 scans were recorded and its average data is presented over the range 4000–400 cm^{-1} as spectra.

ANALYTICAL METHOD

Preparation of Acemannan Standard Stock Solution

20 mg of freeze dried acemannan was weighed accurately and dissolved in 20 ml water to prepare a stock solution. Subsequently, acemannan solution was prepared at different concentration ranging from 100-2000 µg/ml from the stock solution and used for derivatization using congo red dye and used for further analysis.

Preparation of Calibration Curve

Calibration standards were prepared in different concentration ranging from 9.09 to 181.82 µg /ml of acemannan from the stock solutions (100-2000 µg /ml of acemannan) by appropriate dilutions. Plain acemannan solution (100 µg /ml) exhibited λ_{max} of about 220 nm which is not suitable for estimation purpose using UV spectrophotometer. Hence, the prepared calibration

standards were further derivatize using congo-red dye to improve detection sensitivity of aloe polysaccharides. Briefly 400µl of aliquot samples of different concentration of acemannan was transferred into disposable glass culture tubes containing 4 ml Congo red reagent (0.01%w/v) and mixture was subjected to mild vortexing to ensure sample homogeneity. The mixture was kept at room temperature for 20 min. to complete the reaction and absorbance was measured at λ_{max} of 540 nm using UV spectrophotometer [13, 14].

Formulation of Intramural Cream and Preparation of Sample Solution

Aloe vera gel containing cream was formulated using carbopol, glycerin, cetyl alcohol, stearic acid, mineral oil, PEG, *aloe vera* gel and methyl paraben as per the composition reported in Table 1 and evaluated for presence of acemannan by coupling with congo-red dye. *Aloe vera* cream was prepared with different concentration of aloe gel ranging from 10% to 100% w/w. Acemannan was extracted from the cream developed in the lab as well as form marketed *aloe vera* products. Briefly about 10 gm of each cream sample was weighed and quantitatively transferred into polypropylene conical tubes. 20 ml of deionized water was added to each sample, and the mixture was shaken for about 2 hrs for extraction of water soluble polysaccharides i.e. acemannan, each sample was then filtered through a whatman glass microfiber membrane filter. Aliquots of each sample were treated with Congo red reagent and extracted acemannan fraction from the cream was estimated using UV spectrophotometer at λ_{max} of 540 nm [15-17].

RESULTS

Identification of Acemannan by FT-IR Spectroscopy

The FT-IR spectra of acemannan standard sample, isolated freeze dried acemannan and acemannan in gel are shown in Figure 3. The FT-IR spectra showed characteristics bands at 1740 and1250 cm^{-1} which correspond to the C=O and C-O-C stretches of the acetyl groups of acemannan. Characteristic FT-IR bands of acemannan standard as well as isolated samples were in accordance with the reported structure and thus we can ensure that isolated material from the aloe vera gel is acemannan, which explored as phytomarker for development of estimation method for aloe vera gel in marketed formulation [11].

Spectrophotometric Method Development

In present investigation sensitive UV spectroscopic method was developed for estimation of acemannan as a marker for aloe vera gel. Acemannan is complex polysaccharides and gave very less absorbance in UV region and hence its aqueous solution was complexed with congo-red dye to derivatize acemannan to improve its detection at higher λ_{max} i.e. 540 nm (visible range), by UV spectroscopy. The standard acemannan solutions complexed with congo-red dye were scanned in a wavelength range of 200-800 nm in the 2 nm band width against a similarly prepared blank in UV-spectrophotometer. The λ_{max} of acemannan – congo red complex was found to be 540 nm and the same wavelength was used for all the measurements. The overlay UV spectra of acemannan–congo red complex with different concentration of acemannan complexed with congo red is represented in Figure 7. In present investigation the rationale for use of congo red as complex forming agent was its ability to give coloured complex with acemannan and improved estimation sensitivity of acemannan. Congo Red (sodium 4, 4'-diphenyl-2, 2' diazo-bis-1-naphtlamino-4-sulfonate; Figure 4) is an indicator primarily used for estimating free mineral acids and also for staining biological samples. The λ_{max} of Congo red in 1 % (w/v) aqueous solution is approximately 488 nm. The results showed that co-action of the electrostatic and hydrophobic interactions between Congo red and acemannan contribute to acemannan–Congo red chromogenic complex reaction, which was used to quantify the bioactive aloe polysaccharides and give maximum absorbance at 540 nm. The results of the study indicated that intensity of colored acemannan–congo red complex is gradually increased with increased concentration of acemannan and hence intensity of colored acemannan–congo red complex was selected as a estimation parameter which is directly proportional of amount of polysaccharide present in the sample [1, 2, 17, 18].

Linearity

Linearity range was determined by plotting the absorbance at 540 nm (λ_{max}) versus sample concentration extracted from the aloe vera gel as per the procedure described in earlier section. In this method absorbance versus concentration plot is rectilinear over the range of 9.09µg/ml-181.82 µg/ml as per the data represented in Table 2 and Figure 6.

Method Repeatability and Precision

The repeatability and precision of the method was investigated with respect to repeatability. For intra-day precision, standard solution of fixed concentration was analyzed at various time interval and %RSD was noted (limit %RSD<2.0%) and the inter day precision was studied by taken the absorbance of the same concentration of standard solution at various days and the %RSD was calculated (%RSD<2.0%) as shown in Table 3 (a), (b) and (c).

Method Specificity and Validation

The specificity of the method was conducted to prove that the developed method specifically determine the constituent of interest and it is free from determined interference of solvent and other common polysaccharides which might present in aloe vera gel. This is evidenced by the lack of absorbance of other polysaccharides at the specified λ_{max} (540 nm) of acemannan indicating exclusive specificity of method for the estimation of acemannan. The results of the specificity study are reported in Table 4. Similarly developed method for acemannan is validated as per ICH guideline Q2b and results of validation parameters are represented in Table 5.

Estimation of Acemannan in Lab Scale Developed Cream and Marketed Formulation

The applicability of the proposed UV Spectrophotometric method for the assay of acemannan as an estimation marker for aloe gel in cosmetic formulation was examined by analyzing different marketed as well as in house developed cream containing different concentration of aloe gel and the results were demonstrated in Table 6. The results obtained were in good agreement with the label claims for lab scale developed cream and marketed formulation 2, whereas marketed formulation 1 showed presence of only 42 % of active compound with reference to label claim. The results of the assay were reproducible with low standard deviation. The results of analysis of the commercial cosmeceuticals and the recovery study of acemannan suggested that there is no interference of other polysaccharides, aloe vera gel constituents or other polymers which are commonly present in cosmetic preparations.

DISCUSSION

The major chemical components of *Aloe vera* gel are acemannan, free monosaccharides, proteins, amino acids, sterols, organic acids and minerals. The current method is based on the molecular interaction between acemannan and Congo red (coloring agent). The procedure is same as prescribed earlier by Elamthruthy et al., with minor modification in sample preparation [13]. In current investigation pH of the acemannan sample solution was maintain at neutral level (6.5-7.0) rather than alkaline side as reported in earlier literature by Elamthruthy et al. Maintenance of neutral pH will stabilize the coloured acemannan–congo red complex for a longer period of time than the method describe by Eberendu and McAnalley [7]. In present method extended calibration curve ranging from 100 to 2000 µg/ml (9.09 to 181.82 µg/ml in the test solution) was developed with a correlation coefficient of 0.9942/0.9900, Limit of detection (LOD) 2.55 µg/ml and Limit of quantification (LOQ) 8.51 µg/ml. Three formulations were tested i.e. Formulation 1, Formulation 2 and lab scale developed cream; % assay of aloe polysaccharide with standard deviation was found to be 42.07 ± 3.60, 92.84 ± 0.175 and 96.10 ± 0.08 respectively. The assay accurately demonstrated the amount of true aloe acemanan in the samples tested. Results indicated that the average recovery of acemannan found to be 90-96%. Specificity check for method showed that method is specific for determination of acemannan and it was proved that congo-red not formed chromogenic complex with other polysaccharides which are used as potential adulterants, whereas former analytical method like size exclusion chromatographic for estimation of acemannan was based on principle of determination of molecular weight of compound which does not exclude the possibility of presence of adulterant with same molecular weight like acemannan. Experimental results showed that only acemannan showed absorbance at λ_{max} 540 nm. This indicated that the method is specific for determination of acemannan. Overall it was observed that developed colorimetric assay can more reliably detect the true amount of Aloe Polysaccharides i.e. acemannan in the sample and hence acemannan can be routinely explored as potential phytomarker for quality control checks for formulation claiming presence of aloe vera gel [19-21].

CONCLUSION

The experiment showed a binding interaction of acemannan and Congo red by a UV spectrophotometric method. The aggregation extent of aloe acemannan-Congo red chain is the essential factor, affecting the hydrophobic interaction between Congo red and acemannan molecules. The method is based on the interaction of the acemanan with Congo red dye to form a characteristic complex that is proportional to the amount of acemanan in the test sample. High recoveries showed that the method is free from any interference from other compound, present along with acemannan (phytomarker) in the gel. This acemannan–Congo red spectroscopy assay method was considered to be advantageous due to its reliability, simplicity, specificity, sensitivity, and rapidity that can be employed as economical quality control method for *aloe vera* products. Thus, acemannan can be used as a marker in standardization of *aloe vera,* which leads to a new approach in the fingerprint analysis of the *aloe vera* products with an easy day-to-day characterization through UV spectroscopy.

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