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Original Contribution

DEVELOPMENT, STANDARDIZATION AND VALIDATION OF ANALYTICAL METHOD FOR QUALITY ASSURANCE AND QUALITY CONTROL OF *ALOE*-BASED PHARMACEUTICALS AND NUTRACEUTICALS

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ABSTRACT

Pharmacological activity of *Aloe vera* is known for hundreds of years, but a precise and well-established characterization method for all kinds of pharmaceutical formulations is still a challenging task. In the present study, a simple, user- friendly, sensitive, precise, accurate, robust and reproducible method has been developed based on reverse phase-high performance liquid chromatography (RP-HPLC). The RP-HPLC method has been developed, standardized and validated utilizing the *Aloe* marker compounds *viz.*, Aloin A, Acemannan and *Aloe*-emodin that is present in various *Aloe vera* varieties. The total polyphenolic content (TPC) and total flavonoid content (TFC) were estimated spectrophotometrically and an *in-vitro* antioxidant study was also performed to standardize the potential of *Aloe vera* using assays *viz.* DPPH (2,2- diphenyl-1-picrylhydrazyl radical scavenging), NO (nitric oxide) scavenging potential, FRAP (ferric reducing antioxidant power) and TAC (total antioxidant capacity). Simultaneously, tocopherol acetate was also estimated in commercially manufactured pharmaceutical products with the help of the previously standardized HPLC method in our laboratory. Separation of the singular active ingredient of *Aloe vera* was achieved by using an isocratic mode of acetonitrile and water (70:30 v/v) by using a reversed-phase C18 column as stationary phase in a high-performance liquid-chromatography system employing photodiode array detector (PDA plus detector) with a flow rate of 1.0 ml/min. The detection limit of active compound of *Aloe* species was found to be in the range of 0.00020 to 0.00051 μgL^{-1} (20 µL injection of each for five times). The quantitative method of *Aloe vera* extracts standardized vis-à-vis both with peel (*AL-P*) and without peel (*AL-WP*) form gives robust, precise (% RSD 1.13-3.84) and accurate results. This method is suitable for the detection of major pharmacologically active compounds present in *Aloe vera*-based pharmaceuticals and nutraceuticals.

Key words: Lyophilized *Aloe vera* powder; RP-HPLC; isocratic method; rapid and time-efficient analytical method; *in-vitro* antioxidant study; tocopherol acetate; topical cream

INTRODUCTION

Aloe vera (L.) Burm. (*Aloe barbadensis Miller*) is a well-known all-purpose medicinal plant, widely known as a 'healing plant'. It has been used since centuries for the treatment of burn wounds, thermal injuries, chilblains, frostbite and other skin ailments.

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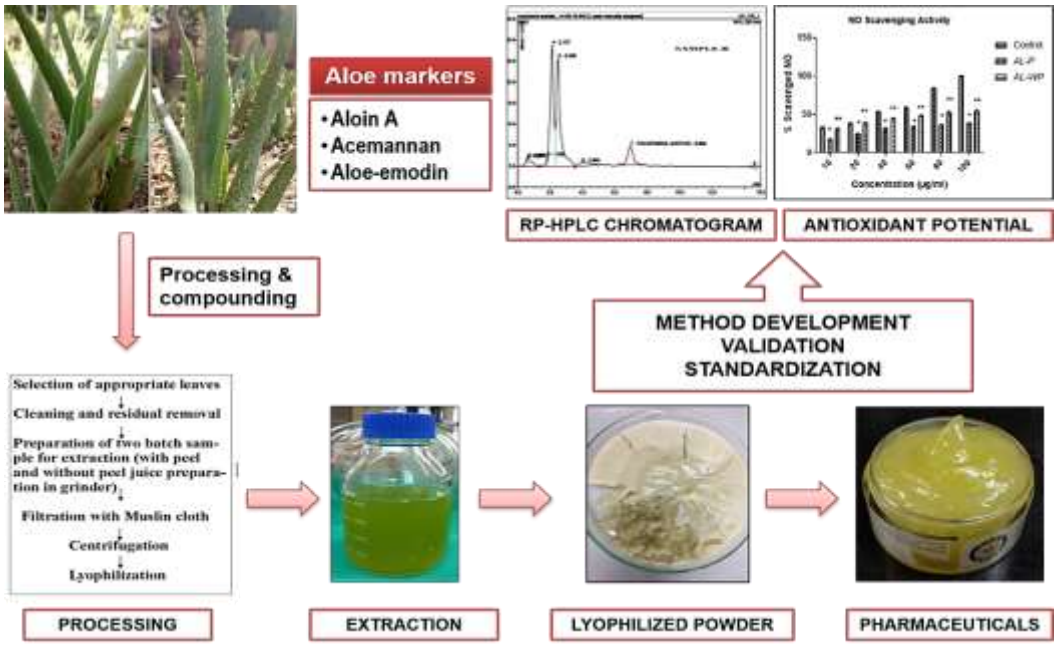
More than 400 species of *Aloe vera* belonging to Asphodeiaceae family are known in which *Aloe barbadensis, Aloe curacao, Aloe arborescens* and *Aloe ferox* are some of the varieties used for medicinal purposes, which require dry subtropical and tropical climates (1). The significant effects of *Aloe* in the treatment of many diseases like cancer, peptic ulcer, diabetes, and inflammation have mainly been attributed to the presence of many rich and pharmacologically active substances such as anthraquinones, polysaccharides, acemannan, vitamin A, vitamin B-12, Vitamin C, Vitamin E and folic acid and several other trace elements, which are generally present in the *Aloe* leaf pulp

(3-4). *Aloe vera* is also known to possess inhibitory activity for Thromboxane A2 (TXA2), which is known as a prominent chemical mediator that causes progressive tissue injury and inflammation mediation via oxidative stress (2).

Some of the major bioactive compounds present in *Aloe vera* whole leaf are depicted in **(Figure 1),** showing OH as a major functional group available for the binding site for their therapeutic activity.



Graphical abstract representing overall processing steps and method development for a complex mucilaginous extract *Aloe vera* (L.) Burm. (*Aloe barbadensis Miller*)

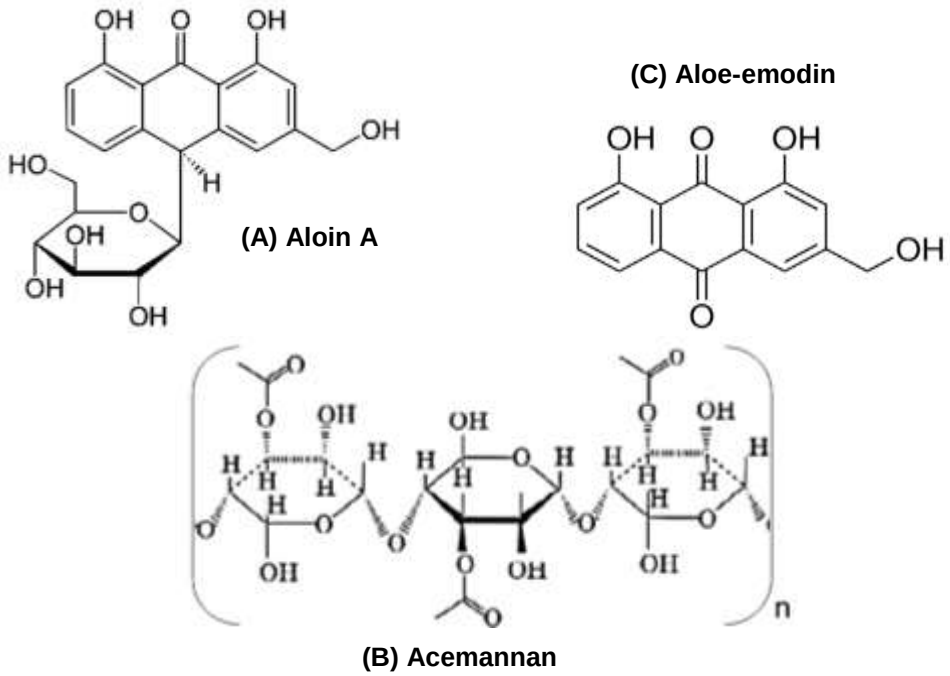


Figure 1. Chemical structure of major bioactive compounds present in *Aloe vera*; (A) Aloin A, (B) Acemannan and (C) *Aloe*-emodin

Aloin A is a series of glycosides known as anthraquinone, which is the bitter principle present in *Aloe vera*, mainly in the peripheral region of the leaf. Anthraquinones (Aloin A, Aloin B and *Aloe*-emodin) are basically phenolic compounds present in the *Aloe vera* leaf sap and appear as yellow or yellow to brown in color. Anthraquinone as Aloin A is a powerful laxative and can cause stomach cramps, thus it should be avoided to be used in children below 12 years as a laxative (5-6). Topical agents contaminated with anthraquinones causes skin itching and blister formation to the very sensitive skin (6-7), thus concentration of aloin should not be exceeded to the recommended limit reported in Ayurvedic Pharmacopeia (8).

Acemannan is a well-known active principle of *Aloe vera* that exhibits accelerated wound healing and reduces skin damages caused by radiation through macrophage activation and also stimulated release of fibrogenic cytokines factors and which upon binding to acemannan prolong stimulation of granulated tissue (9-11).

From basic characterization and pre-formulation to the formulation development to the end products many studies such as identification test, compositional study, cumulative drug release study, active pharmacological constituent identification, and end product efficacy, bioavailability and stability etc. have to be carried out by using chromatographic techniques as HPLC/HPLC-MS, GC-MS, and HPTLC. Since these techniques are easy and feasible they find use in pharmaceutical industries for characterization of the critical parameters. In this study, we have designed and developed an easy and feasible technique for the characterization of *Aloe vera* gel, powder, and/or pharmaceutical products by characterizing some of the most active pharmacological constituents such as Aloin A, Acemannan, and *Aloe*-emodin by using an HPLC with a very common solvent system in a single run time.

Simultaneously, a separate study was carried out to establish the presence of tocopherol acetate in commercially available topical formulations of *Aloe vera*. Quantitative determination of tocopherol acetate was carried out with the help of already established laboratory protocols for the estimation of tocopherol acetate in various products. Since commercial topical formulations such as emollients, ointment, lotion, juice and other therapeutic and beauty products contain several active constituents of *Aloe vera* in different ratios, it is very difficult to characterize them vis-à-vis the bioactive constituents. The developed method is able to do so quantitatively in a precise manner.

MATERIALS AND METHODS

1 Standards and chemicals

The standards viz., Aloin A, acemannan and *Aloe*-emodin were received as a gift from Prof. K.S. Laddha of the Institute of Chemical Technology (Deemed to be University; MHRD/UGC), Nathalal Parekh Marg, Matunga, Mumbai - 400019, Maharashtra, India). 2-2-Diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu, napthylethylenediamine dihydrochloride, 2,4,6-tripyridyl-s-triazine, sodium nitroprusside, aluminum chloride, sodium hydroxide, sodium carbonate, ferric chloride hexahydrate, ferrous sulphate, sodium phosphate, ammonium molybdate was purchased from Sigma Aldrich (St. Louis USA). Ascorbic acid, rutin trihydrate, tocopherol acetate, acetonitrile (HPLC grade) and ethanol (HPLC grade), were also purchased from Sigma Aldrich (St. Louis USA), sulphuric acid (analytical grade) and chloroform was purchased from Merck with the marked purity grade of 95%. Extra ultra-pure water with 18.2 MΩ resistivity was obtained from Milli-Q apparatus (Merck ELIX®) and used for mobile phase development system as well as for the entire analysis of the proposed method development of *Aloe vera* in pharmaceutical topical formulations. Stock solution for each standard was prepared in methanol and stored at 4^{o} C for carrying out each successive analysis and sample was extracted by the earlier established method and further dissolved in methanol to measure the concentration of each pharmacologically active substance present with respect to the choice of standard used in the method development. Further, all the methods were successively performed thrice to maintain accuracy in the test sample.

2 Preparation of lyophilized powder of *Aloe vera* leaves (with and without peel)

Aloe vera whole leaf juice extract (with peel and without peel) was extracted by the previously reported method (3, 13-15), with few modifications. Briefly, an aged *Aloe vera* plant grown in DIPAS of more than three year of age was selected for the study. Measurement of leaf was done by using a ruler to obtain a uniform leaf size so that possibility of residual changes in concentration of active pharmacological material could be minimized. After selecting an appropriate

leaf size for *Aloe vera* extraction, the leaves were washed thrice with normal tap water, followed by distilled water and then rinsed with 30% chloroform to remove any unwanted debris present upon the outer surface of the *Aloe* leaf. After rinsing the whole leaf, *Aloe* leaf was kept at room temperature to completely dry, after taking pre-weight of the *Aloe* leaf, the *Aloe* leaves were divided into two parts, for whole leaf extraction i.e., with peel (*AL*-P) and without peel (*AL-WP*) *Aloe vera* leaf juice. The juice was obtained by grinding method and then filtered using a muslin cloth and then centrifuged by using an Ultracentrifuge system (Rota 4R-V/Fm Plasto Crafts) at 4^{o}C and 4500 rpm for 10 min. Then the final juice was collected and immediately loaded for lyophilization by using a pre-freezing condition of -20^{o}C (Allied Frost FD-5 lyophilizer) and vacuum condition was maintained at -60^{o}C followed by drying cycle, maintained at 30^{o}C. After achieving the final temperature, the lyophilized products were immediately collected and packed in an air-tight container, and used for analysis.

3 Total Polyphenolic content and Total Flavonoid content studies of *AL-P* and *AL-WP*

Total polyphenolic content (TPC) and Total flavonoid content (TFC) was quantitatively determined in the lyophilized *Aloe vera* powders in both with peel and without peel extract.

TPC was determined by a universally accepted method named Folin-Ciocalteu Reagent (FCR) method described by Sharma et al., (2016). Briefly, a concentration range of 5μg/mL to 45 μg/mL of standard gallic acid, lyophilized *Aloe vera* powders (*AL-P* and *AL-WP)* of 1mg/ml was mixed with 10 mL of Milli-Q water and subsequently, each dilutions was then mixed with 1 mL of FCR. After 5 minutes of incubation in dark, 2 mL of 20% (w/v) sodium carbonate solution was added to the above mixture. Again the mixture was incubated in dark for 60 minutes after which the absorbance for TPC was recorded at 750 nm on UV/Visible spectrophotometer 6705 (Jenway). Each experiment was carried out in triplicate and calculated result was expressed in mg of Gallic acid equivalents (GAE)/g of dry weight of extract (DWE).

TFC in *AL-P* and *AL-WP* was quantitatively determined by using aluminum chloride method previously described by Zhishen et al., (1999). Briefly, a standard stock solution of rutin trihydrate (RT) with concentration range of 10 μg/mL to 300 μg/mL or test solution of AL-*P* and *AL-WP* (1 mg/mL) was prepared in 4 mL of Milli-Q water. Subsequently 0.3 mL of 5% (w/v) sodium nitrite solution was added to the above solution and incubated for 5 minutes in dark. Then 0.3 mL of 10% (w/v) aluminum chloride (freshly prepared in dark before use) and 2 mL of 1M solution of sodium hydroxide was added to the mixture and then total volume was made up to the 10 mL with Milli-Q water. Each sample was prepared in triplicate and absorbance was recorded at 510 nm by using UV/Vis spectrophotometer 6705 (Jenway). The TFC in lyophilized *AL-P and AL-WP* was calculated and expressed in mg of Rutin trihydrate equivalents (RTE)/g of dry weight of extract (DWE).

4 *In-vitro* antioxidant potential of lyophilized *Aloe vera* powder

In-vitro antioxidant potential of lyophilized *AL-P* and *AL-WP* was performed by various in- vitro methods *viz.* DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging potential, FRAP (Ferric Reducing Antioxidant Power) assay, NO (Nitric oxide) scavenging activity, and TAC (Total antioxidant capacity) assay.

DPPH free radical scavenging potential of *AL-P* and *AL-WP* was determined by the procedure reported by Uddin (2008). Briefly, 3.0 mL of 0.1mM methanolic solution of DPPH was prepared and added to the standard solution of ascorbic acid (0.2 mL) with a different concentration range of 10 μg/mL to 100 μg/mL and concentrations range from 100 μg/mL-1000 μg/mL were selected for up to 50 % of total inhibition for *AL-P and AL-WP*. All dilutions were prepared in triplicate and incubated in dark for 30 minutes at room temperature and the absorbance was recorded at 517 nm on UV/Vis spectrophotometer 6705 (Jenway). The percentage inhibition (%I) of DPPH free radical was calculated by using the following equation given below:

% I [DPPH Free Radical] scavenging potential = [(Ac-As)/Ac] x 100

Where, Ac is the absorbance of the control i.e., [DPPH solution] and As is the inhibited absorbance of the samples/standards.

The % inhibition for each standard and sample was calculated with respect to the concentration and the result was correlated with standards.

NO (Nitric Oxide) scavenging potential activity was measured by the method described by Hazra et

al., (2008). Briefly, different concentration of the standard (ascorbic acid) and test samples (*AL-P and AL-WP)* in the concentration range of (10 µg/mL to 100 µg/mL) was mixed with 0.8 mL of 10 mM sodium nitroprusside solution previously prepared in phosphate buffer saline (pH 7.4). Then the sample mixture was incubated at 40^{o}C in dark for 150 minutes. 1 mL of 0.33 % sulphanilamide, prepared in 20% of glacial acetic acid), was added to the solution mixtures and incubated for 5 min in dark at room temperature. Subsequently, 1 mL of napthylethyenediamine dihydrochloride (0.1% w/v) solution was added and further incubated for 30 minutes in dark at room temperature. A light pink colored chromophore was developed in the standard and test sample due to scavenging of NO, the observation was recorded at 540 nm on UV/Vis spectrophotometer 6705 (Jenway). The percentage inhibition (%I) of NO free radical was calculated using the equation described below:

%I [NO] free radical activity = [Ac-As/Ac] x 100

where, Ac is the absorbance of control and As is the absorbance of samples/standard solutions.

FRAP (Ferric ion Reducing Antioxidant Power) assay of *AL-P*, *AL-WP* and standard (Ferric sulphate) was measured according to the method reported by Benzie et al. 1996. Briefly, a standard stock solution with concentration range of 50 µg/mL to 500 µg/mL and test solution (1 mg/mL) each was prepared and were mixed with 2.8 mL of FRAP solution [300mM acetate buffer (25mL), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCL (2.5 mL) and 20 mM $FeCl_3.6H_2O$ (2.5 mL)]. After which the whole solution in triplicate were incubated for 30 minutes in dark. A standard plot was drawn and result was expressed as mg of ferrous II equivalent Fe(II)E/g of dry weight of extract DWE.

Quantitatively, TAC (Total antioxidant capacity) assay was performed according to the method used by Prieto et al. (1999) i.e., phosphomolybdenum complex formation method. Briefly, a standard (ascorbic acid) solution with a concentration range of 10 µg/mL to 600 µg/mL, and test samples (*AL-P and AL-WP)* of 1 mg/mL was prepared in Milli-Q water. 0.3 mL of each sample was mixed with TAC reagent solution composed of 3 mL; 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. Then the entire sample was incubated at 95^{o}C for 90 minutes. The samples were kept aside for cool down at room temperature and then absorbance was recorded at 695 nm by using UV/Vis spectrophotometer 6705 (Jenway). The results were expressed in mg of ascorbic acid equivalent (AAE)/g of the dry weight of extract DWE.

5 Instrumentation and chromatographic conditions

A Thermo scientific fisher Ultimate 3000 series of UHPLC system equipped with Ultimate 3000 dual gradient pump, an auto-sampler unit and a diode array detector of 3000 Ultimate series perfectly aligned with the instrument was used for analysis. The chromatographic method was developed by using Thermo Scientific column (Acclaim^{TM} Polar Advantage, USA) C18 having a pore size of 5µm with a dimension of 4.6 x 250 mm supported with a guard column 5µm having 4 x10 mm diameter (Cranbury, NJ, USA). The chromatographic conditions were maintained throughout the process by using a mobile phase of acetonitrile: water (70:30 v/v), column temperature 38^{o} C and flow rate of 1.0 ml/min, and each injection volume set used 20µL/ per injection.

6 Preparation of standard stock solution

Standard stock solution for each compound i.e., Aloin A, Acemannan and *Aloe*-emodin was prepared in methanol, and stored at 4^{o} C for analysis. A 0.25 mg/ml solution of Aloin A, 2.0 mg/ml solution of Acemannan and 2.0 mg/ml solution of *Aloe*-emodin was prepared. The concentration was decided by a random single run of each standard and by means of each successive peak height i.e. peak area for the standard compound and then the obtained proportion of standard mix, was set for method development.

7 Sample preparation method for the study of standard marker compounds present in *AL-P, AL-WP* and *Aloe*- based pharmaceuticals

Lyophilized *AL-P*, *AL-WP* and topical pharmaceuticals (sample A and sample B) were extracted by the method reported in previous literature for aloin and other related compound found in *Aloe vera* leaf as an active pharmacological compound (12-13, 22) and for identification of marker compound (s) in the pharmaceutical formulation, a method reported by Kaliyaperumal et al., (2013) was opted with a slight modification and trials were done for each active compound. The method was lead to increase in yield and more extractive values than the simple methanolic extract reported in previous articles which are most commonly used for analysis. Briefly, 10 mg (weight equivalent of 200x concentration of total juice) weight of *AL-P and AL-WP* was soaked in 60% v/v ethanol;

subsequently 1gm of each topical pharmaceutical (containing 50% w/v *Aloe* juice in each) was also soaked in 60 % v/v ethanol for 24 hrs in dark at room temperature. Then above samples were centrifuged at 4500 rpm for 8 min at 4^{o} C and then the supernatant was collected and an equal volume of sulphuric acid and chloroform of 1:1 v/v was added to it. Then this mixture was vortexed for 5 min to obtain a homogenous mixture. Then the final mixture was refluxed at 50^{o} C by using Rota evaporator (BUCHI-R-300), equipped with interface I-300, heating bath- B-300 base, Vacuum pump- V-300 along with Recirculating chamber-F-305. A final extract material of yellowish-brown colloids was obtained and used for further analysis by RP-HPLC.

8 Sample preparation methods for the analysis of tocopherol acetate in topical pharmaceuticals

The sample method for tocopherol acetate from the topical formulations was done with a very simple method according to the protocol established in the laboratory. Briefly, 500 mg of each given formulations (A, B and C) claiming a proportionate amount of tocopherol acetate in the formulations, was extracted by using HPLC grade methanol (any chemical or other sample preparation was avoided due to its highly oxidizing nature). Filter a little volume of methanol was added to each sample and kept on the sonicator for 1 hr. and then viscous material was filtered out using Whatmann filter paper no. 1 and then the filtrate was transferred to the 25 ml volumetric flask and further volume was made up to the 25 ml with methanol. The obtained solution was then vortexed for 10 minutes and then centrifuged at 4^{o} C on 4000 rpm for 10 minutes, the supernatant was collected and filtered by using a syringe filter of 0.45 micron pore size before injecting the sample to HPLC.

9 Method development and validation

Method validation assays were carried as per the guidelines of the United States Pharmacopoeia (USP32) established under the United States Pharmacopeial Convention 2009 (23).

9.1 System suitability

System suitability analysis for the *Aloe* standard viz. Aloin A, Acemannan and *Aloe* emodin were carried out by injecting the standard stock solution of the same concentration five times and peak spiking with respect to area under the curve was observed after each successive run. The acceptability of % Coefficient of variation (%CV) of the standard peak area and retention time for the same run were accepted to the limit of ±5% variation.

9.2 Linearity range of standards (Calibration curve)

To construct linearity range of each standard, the standard mixture was run at different absorbance maxima i.e., 210 nm for Aloin A, 220 nm for Acemannan and 254 nm for *Aloe* emodin with the concentration range of 0.05, 0.15, 0.25, 0.35 and 0.55 mgL^{-1} (Aloin A), and concentration range of 0.25, 0.50, 1.0, 1.5, and 2.0 mgL^{-1} (Acemannan and *Aloe* emodin).

9.3 Limit of Detection and Limit of Quantification

Limit of detection (LOD) and Limit of quantification (LOQ) i.e. sensitivity of *Aloe* standards mixture was estimated by standard chromatographic system suitability method. LOD is basically defined as the lowest concentration of the analytes in the study which may be detected by the instrument used which is represented as three times average of baselines noise. Whereas, LOQ is the unit which represents the least amount of analytes which is detected by instrument provided a signal-to-noise ratio of over 10 with the precision limit (% CV) and accuracy (% biasness) at each successive run within their acceptability range of (10 %) (24).

9.4 Specificity and Selectivity

The *Aloe vera* is itself a very complex and mucilaginous material and thus has a complex matrix as compared to other natural extracts. Therefore, testing of specificity and selectivity for natural extract material becomes an important parameter. This method was developed by using the isocratic system and specificity for each analytes of *Aloe vera* in the matrix was examined, specificity gives detection of a single compound with precise resolution in a developed method for complex matrices. Similarly, selectivity is the result of chromatographic separation of each of the individual analytes in the given mixture without any peak interferences and interaction and thus represents reliability of the selective method.

RESULTS AND DISCUSSION

1 Total Polyphenolic content and Total Flavonoid content *AL-P and AL-WP*

Aloe vera as a medicinal plant possesses many active phytoconstituents, but due to their existence of wide variety, antioxidant potential of *Aloe vera* may vary from plant-to-plant and thus this becomes important to estimate the activity of plant

material before making any pharmaceutical product was used for any therapeutic effect. The plant possesses many phenolic compounds which are known as very good antioxidant agents as well as act as chelating agents. The presence of phenolics and flavonoids were experimentally assessed by Folin-Ciolteu Reagent (for polyphenolic compounds) and aluminum chloride method (for flavonoid compounds). The result of *AL-P and AL-WP* differed slightly and these differences may be due to several reasons one of them may be the age difference between the two plants. TPC of both lyophilized *AL-P* and *AL-WP* was found as 2.434±1.47 mg GAE/g DWE and 7.673±0.85 mg GAE/g DWE respectively. TFC was estimated as follows 6.667± 0.76 mg RTE/g DWE and 8.466± 0.680 mg RTE/g DWE, graphically this has shown in **(Figure 2),** Alike result was also reported by Wariyah et al., (2016).

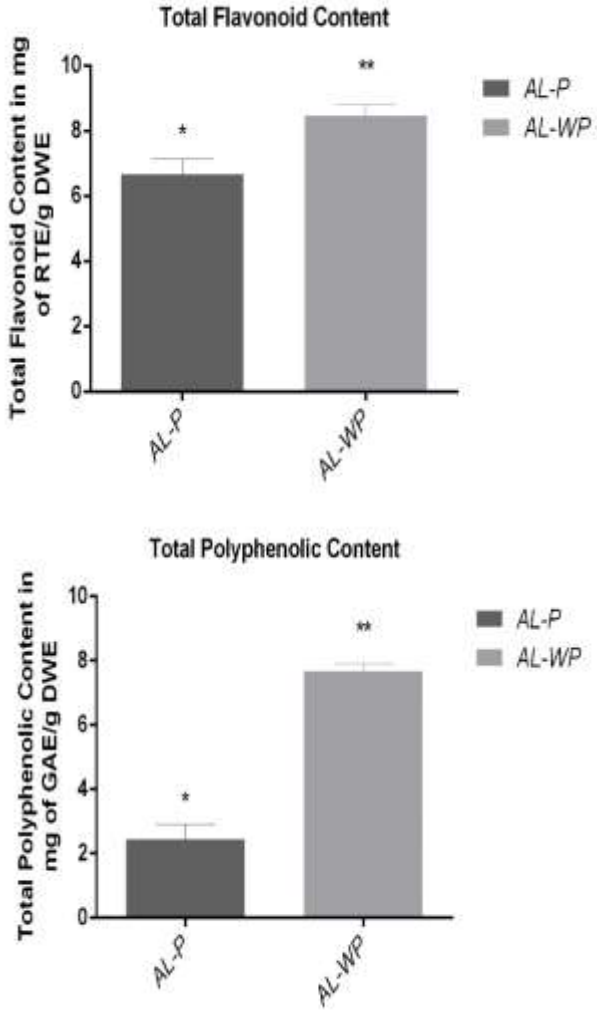
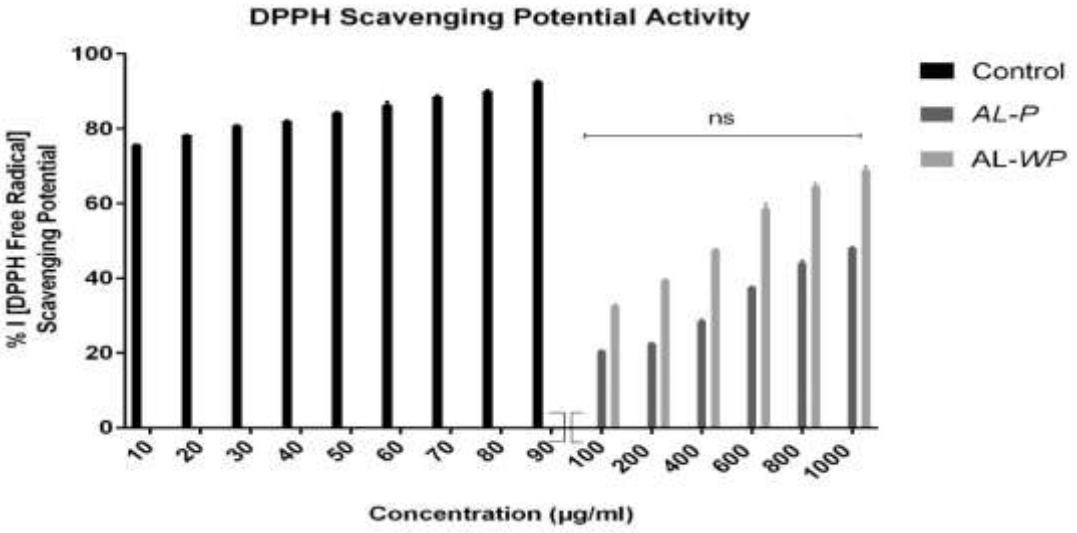
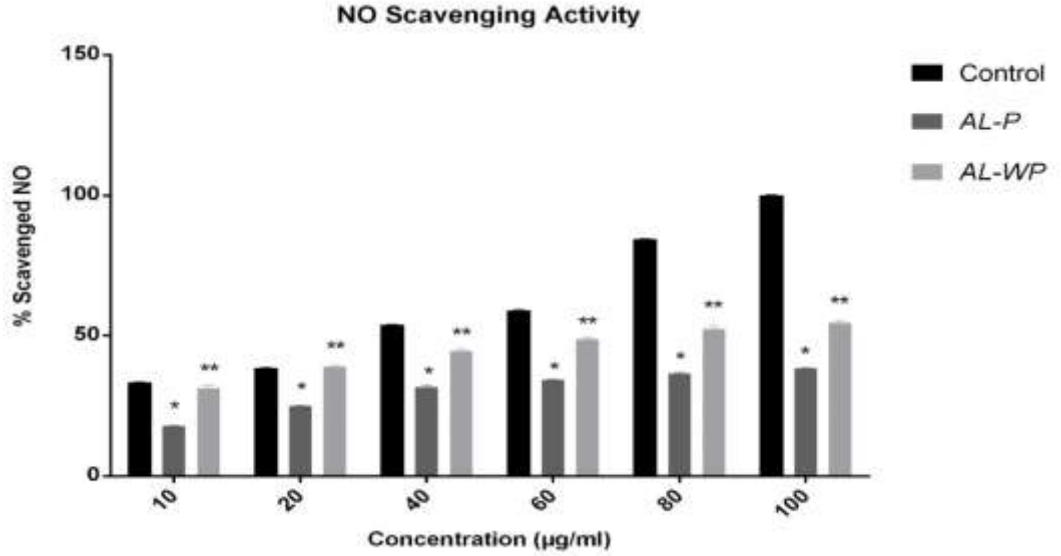


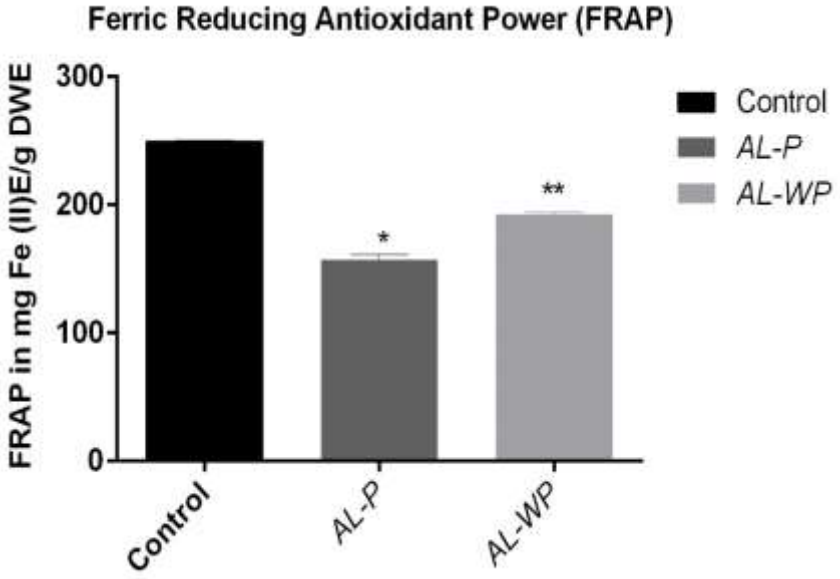
Figure 2. TPC and TFC of lyophilized *AL-P* and *AL-WP* against a positive control as gallic acid and rutin trihydrate respectively; results are presented as mean ± SEM (n=3), using t-test statistic and significant values are represented as: **p-value <0.01 and *p-value <0.05.

2 *In-vitro* antioxidant potential of *AL-P* and *AL-WP*

DPPH free radical scavenging capacity of the both extract was determined by methanolic DPPH solution. DPPH activity was assessed for significant inhibition capacity, after which the results were statistically analysed by applying One-way ANOVA using multiple comparison method, and found to be insignificant with respect to standard compound L. Ascorbic acid. The maximum percentage inhibition of DPPH of *AL-P* and *AL-WP* was found to be 48.12% and 68.88 % at 1mg/mL concentration respectively as shown in **(Figure 3).**


DPPH Scavenging Potential Activity
% I [DPPH Free Radical] Scavenging Potential
100
80
60
40
20
0
ns
Control
AL-P
AL-WP
10
20
30
40
50
60
70
80
90
100
200
400
600
800
1000
Concentration (µg/ml)


NO Scavenging Activity
% Scavenged NO
150
100
50
0
Control
AL-P
AL-WP
*
**
10
20
40
60
80
100
Concentration (µg/ml)


Ferric Reducing Antioxidant Power (FRAP)
FRAP in mg Fe (II)E/g DWE
300
200
100
0
Control
AL-P
AL-WP
*
**

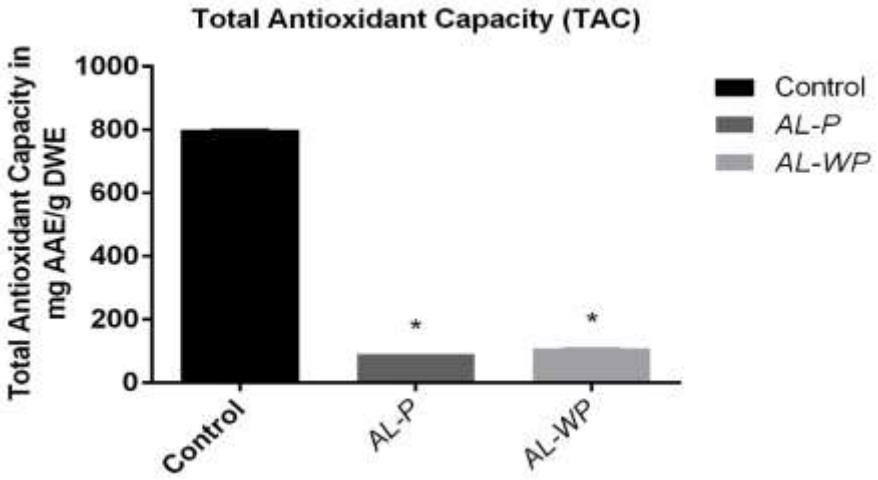


Figure 3. DPPH, NO, FRAP and TAC of lyophilized *Aloe vera* powder against a positive control of L-Ascorbic acid for DPPH, NO and TAC and $FeSO_4$ for FRAP respectively; Result presented as mean ± SEM (n=3), One-way ANOVA using Tukey's multiple comparison test statistics and significant values are represented as: **p-value <0.01 and *p-value <0.05, where; ns* is non-significant.

Nitric oxide in combination with free superoxide contributes to cellular damage due to oxidative stress in tissue, thus for healing mechanism in wounds it becomes necessary that the material should possess enough scavenging potential to accelerate wound healing. In this study, NO scavenging *in-vitro* potential of *AL-P* and *AL-WP* was performed with the percentage inhibition range from 10 µg/mL to 100 µg/mL up to the maximum percentage of inhibition range of *AL-P* and *AL-WP* with respect to standard compound and it was found to be 38.43% at 0.1 mg/mL and 54.55 % at 0.1 mg/mL. The obtained results were statistically analysed by using One-way ANOVA with Tukey's multiple comparison method and results were expressed with the p-value <0.01 and <0.05, graphically depicted in **(Figure 3).**

FRAP (Ferric ion Reducing Antioxidant Power) assay of *AL-P and AL-WP* was determined by the ability of *Aloe vera* to reduce the 2,4,6-tripyridyl-*s*-triazine (TPTZ)-Fe(III) complex to 2,4,6-tripyridyl-*s*-triazine (TPTZ)-Fe(II), which gives blue color after reduction of the complex with *Aloe vera.* The FRAP activity of lyophilized *Aloe vera* A and *Aloe vera* B was found to be 156.83±0.659 mg Fe (II)E/g DWE and 192.66±1.416 mg Fe (II)E/g DWE respectively. One-way ANOVA with Tukey's was used to predict the significant difference with control and it was found that results with both of the *Aloe vera* powder were significant with the p-value <0.01 and <0.05; whereas; *AL-P* exhibited significantly lower FRAP activity with control in comparison to *AL-WP*, which are graphically depicted in **(Figure 3).**

Phosphomolybdenum complex formation method was used to study the total antioxidant capacity (TAC) in *AL-P* and *AL-WP*. TAC of both the sample ranged between 89.66±0.577 mg AAE/g DWE and 108.66±1.000 AAE/g DWE. TAC of L-ascorbic acid was found to be much higher than both of the powder in the present study, but was significant with p-value of <0.05 and result is depicted in **(Figure 3).**

3 Choice of method development and experimental conditions

The popularity of natural products amongst the consumers for use as food, cosmetics, and therapeutics has increased; the acceptance rate for the natural products has increased leading to consumer satisfaction. However, the characterization and development aspect for the herbal material is much more complex than the synthetic one. Variability in chemical composition, as well as in maintenance of steady therapeutic value is a big challenge with natural plant products. Thus, the characterization of material with some advanced techniques is often recommended herbal formulations. In this study, a well-known plant *Aloe barbadensis* was used as a test material with reference to the standard markers and a fast and reliable method was developed by using HPLC; by utilizing three major compounds in a single run time of 45 min. An isocratic program was developed for the separation of the

compound from complex matrices and found to be reproducible upon each sample analysis.

A symmetric peak with the marked resolution was found under the given chromatographic conditions, earlier researchers have developed methods by means of using methanol instead of acetonitrile, but it has been also reported that methanol causes ionization of the *Aloe* compound (13). In this method, using water and acetonitrile system, similar peak height with respect to concentration was found; thereby suggesting the suitability of the method for *Aloe* and *Aloe*-based therapeutic formulation, including commercial ones.

The standard preparation method was validated for its stability in the carrier vehicle and results were reported as mean of peak area ± RSD (Relative standard deviation; n=5) as depicted in **(Table 1).**

Table 1. *Result from sample preparation methods*

Standards	**Peak Area (mAU*min)**	**%RSD**
Aloin A	262.908	2.02 ± 5.2
Acemannan	97.1430	3.84 ± 4.9
Aloe-emodin	162.358	1.13 ± 2.1

3.1 Limit of Detection and Limit of Quantification

Limit of detection (LOQ) of all the three compounds was found to be within an acceptable range (0.00020 μgL^{-1} to 0.00051 μgL^{-1}) depicted in **(Table 2).** In addition, Quantification limit was appropriate to detect the minimum concentration in the samples.

Table 2. *Linear regression calibration curve representing regression coefficient and linear regression slop of the line*

Components	**Linear Regression line slope**	**Regression coefficient (R^2)**	**Limit of detection (LOD) (μgL^{-1})**
Aloin A	0.5355	1	0.00020
Acemannan	0.0500	1	0.00051
Aloe-emodin	0.0251	1	0.00032

3.2 System suitability

System suitability study of the standard marker wad assessed and is depicted in **(Table 3).** Optimization of HPLC method for determination of these marker compounds in the *AL-P* and *AL-WP*, as well as in the pharmaceutical formulation, was found to be suitable with the following parameters: column temperature: 38^oC; flow rate: 1.0 ml/min; mode of elution was isocratic with overall separation of all the three compounds within 45 min of total run time.

Table 3. *System suitability test results for the optimized HPLC method*

Standard	**Retention Time (min) (%CV)**	**Capacity factor (K')**	**Selectivity (α)**	**Resolution (Rs)**	**Tailing Factor**	**Plate count**	**Mean Peak Area (mAU)**
Aloin A	6.317 ±0.2	1.500	1.97	6.30	1.00	384	262.90
Acemannan	10.082 ±0.4	3.000	5.00	25.2	1.05	660	97.143
Aloe-emodin	40.711±0.6	15.28	7.64	81.42	1.10	1019	162.36

The coefficient of variation in all three standards was found to be within the acceptable range of ±5% variation, thus the system was found to suitable for the developed method.

3.3 Assays linearity range (Calibration curve)

Linearity range (r^2 =1) for all three standards was found satisfactory; the linearity range was established with a concentration range of 0.05-0.55 mgL^{-1} for Aloin A and for Acemannan and *Aloe*-

emodin it was optimized in the range of 0.25-2.0 mgL^{-1}, and no significant change was observed. The linearity range and slop of lines from the calibration curve are depicted in **(Figure 4)** and **Table 2.**

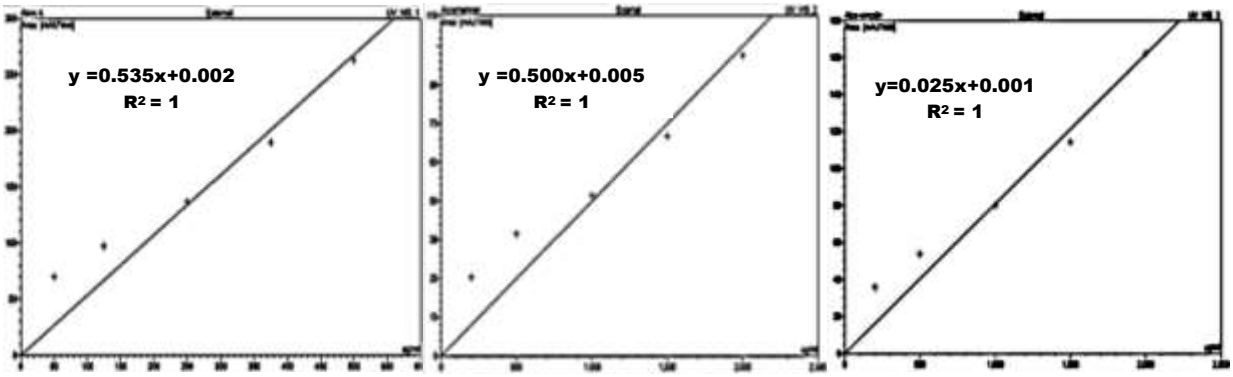


Figure 4. Linearity range graph of *Aloe* standard markers *viz.* Aloin A, Acemannan and *Aloe*-emodin

3.4 Specificity and Selectivity

The standard chromatogram for the developed method was found to be specific and selective in the isocratic mode with the use of a very common elution solvent system i.e., water and acetonitrile system. No ionization of peaks was observed during the analysis and standard run. The standard chromatogram along with test samples is depicted in **(Figs. 5-7)** whereas overlay spectra of *Aloe*-standard Aloin A at 210 nm with retention time (6.317 min.), *AL-P*, *AL-WP* and topical pharmaceuticals (Sample A and Sample B) is shown in **(Figure 5**). It was observed that *AL-P* contains Aloin A, whereas *AL-WP* does not possess Aloin A. Thus, these findings revealed that the perennial part of *Aloe vera* contains Aloin A. Similar findings have been reported earlier by Balaji et al., (2013). Similarly, in the case of pharmaceutical commercial formulation- Sample A, presence of Aloin A (3.6231 µg/ml) was detected and is depicted in **(Figure 5).** Maximum elution absorption of Acemannan with retention time (10.082 min.) was measured at 220 nm **(Figure 6**) and in *AL-WP*, Sample A and Sample B were found satisfactory, but in the case of *AL-WP* amount of Acemannan was below the quantification limit. Similarly, **Figure 7** depicts elution of *Aloe* emodin with retention time (40.711 min.) at absorption maxima 254 nm and *Aloe*-emodin was found satisfactory in *AL-WP* only, which may be due to variance in the *Aloe* species.

The concentration of all samples is depicted in **Table 4.**

Table 4 *Presence of Aloin A, Acemannan and Aloe-emodin in the AL-P, AL-WP and commercial topical pharmaceuticals.*

Standards/Samples	**Concentration of different *Aloe* compound in *Aloe* extract and pharmaceutical formulation (µg/ml)**			
	AL-P (µg/ml)	**AL-WP (µg/ml)**	**Sample A (µg/ml)**	**Sample B (µg/ml)**
Aloin A	8.2470	n.d.*	3.6231	n.d.*
Acemannan	0.0791	n.d.*	1.2674	2.678
***Aloe*-emodin**	0.0009	n.d.*	n.d.*	n.d.*

n.d.* not detected in the proceeded samples

4 Results from the analysis of presence of Tocopherol acetate in the topical formulations

Alpha-tocopherol i.e., vitamin E is the commonly used ingredient in many cosmetic as well as in pharmaceutical and nutraceutical preparations. In marketed *Aloe*-based pharmaceuticals and nutraceuticals many stabilizing and antioxidant agents such as vitamin E, vitamin C and vitamin A are commonly used in *Aloe*-based products (29). Thus, a parallel standard method for QA/QC purpose is needed to be established. In the given formulations that claimed the presence of Tocopherol acetate (i.e., a salt form of vitamin E), the presence or absence of the tocopherol acetate

was quantitatively determined by HPLC using previously established method developed by our laboratory wherein methanol: water (97:3 v/v) was used for quantification of tocopherol acetate in the topical pharmaceutical samples (Sample A, B and C). Standardized protocol for developed method was established with total run time of 15 minutes with the injection volume of 50µL and the flow rate was kept at 1.5 mL/min with the column temperature assisted at 25^{o}C. The retention time of tocopherol acetate was observed at 283 nm, whereas standard RT was found at 7.13 minutes and a similar method was developed by Aly N *et al.* (2010). Since *Aloe* finds use in a large number of pharmaceuticals, cosmeceuticals and nutraceuticals, newer quality control methods are the need of the hour. Quality control of *Aloe vera* (*Aloe barbadensis*) and *Aloe ferox* using the sophisticated band-selective quantitative heteronuclear single quantum correlation spectroscopy (bs-qHSQC) has been recently reported by Girreser U *et al.* (2019). Fujita, M. (2019) have recently reported a newly developed means of HPLC-fluorescence analysis for predicting the skin sensitization potential of multi-constituent substances using Amino acid Derivative Reactivity Assay (ADRA), which is proposed as a chemicoalternative to animal testing for future assessments of sensitization risks to cosmetics, pharmaceuticals and nutraceuticals. In the current study, the sample A and B of the topical formulation approximately claimed amount of the tocopherol acetate as 23.25 µg/ml and 27.41 µg/ml was found respectively, but in the sample C tocopherol acetate was not detected as shown in **Table 5**. The resultant chromatogram for each sample is depicted in **(Figure 8)** respectively.

Table 5 *Determination of total tocopherol acetate present in commercial topical formulation as a stabilizing agent for Aloe vera.*

Ingredient	**Concentration of Tocopherol acetate in topical pharmaceuticals (µg/ml) claiming presence of Tocopherol acetate (Claimed amount is not mentioned)**		
	Sample A	**Sample B**	**Sample C**
Tocopherol acetate	23.25 µg/ml	27.41 µg/ml	n.d.*

n.d.* not detected in the test sample

CONCLUSIONS

The present study was carried out to develop and standardize an RP-HPLC method for the determination of various *Aloe* markers such as Aloin A, Acemannan, and *Aloe*-emodin present in *Aloe vera* and *Aloe*-based pharmaceuticals by employing a user-friendly, simple, sensitive, precise, accurate, robust and reproducible method. Subsequently, total phenolic content (TPC) and total flavonoid content (TFC) was also determined for the lyophilized *Aloe vera* powder but the content was found to be very minimal as compared to other plant material, which are rich in phenolic contents. Simultaneously *in-vitro* antioxidant study such as DPPH, NO, FRAP and TAC was also performed which showed significantly active antioxidant properties of lyophilized *Aloe vera* powders (*AL-P* and *AL-WP)*. Along with this study, the concentration of tocopherol acetate (vitamin E) was also analyzed in the topical pharmaceuticals which are used as a stabilizing agent in *Aloe vera* and *Aloe*-based pharmaceuticals and nutraceuticals. Method development and validation for the standardization of phyto-constituents in the herbal pharmaceuticals and nutraceuticals are very complex and tedious as well as the suitability of the method largely depends upon the specificity, sensitivity, and reproducibility of the developed method. Extraction of the active principle from the swellable matrix like *Aloe* is comparatively very difficult to reproduce in comparison to less complex herbs or non-mucilaginous herbs; as the variety or nature of collection will affect the results which significantly change the concentration of active phytoconstituents with material to material. Thus, it becomes necessary to develop a suitable method with a standardized sample preparation technique where replication of the results in the crude as well as in the *Aloe*-based pharmaceuticals and nutraceuticals is reliable. The method developed, standardized, tested and validated in this study makes it feasible to evaluate the quality of *Aloe vera* species of different origins and can also be used to evaluate adulteration in the marketed *Aloe*-based products. The developed method has been used for characterization of several commercially available formulations and also for quality assurance and quality control of *Aloe*-based pharmaceuticals and nutraceuticals.

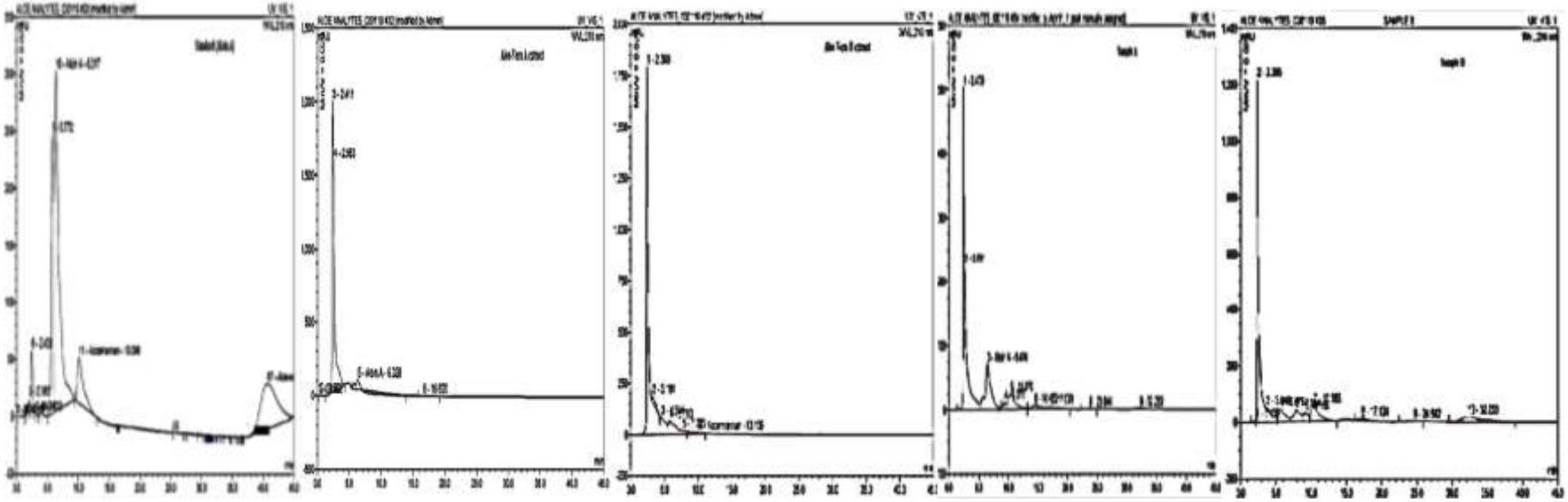


Figure 5. RP-HPLC chromatogram of standard and sample showing presence and absence of Aloin A at 210 nm with retention time of 6.317 min

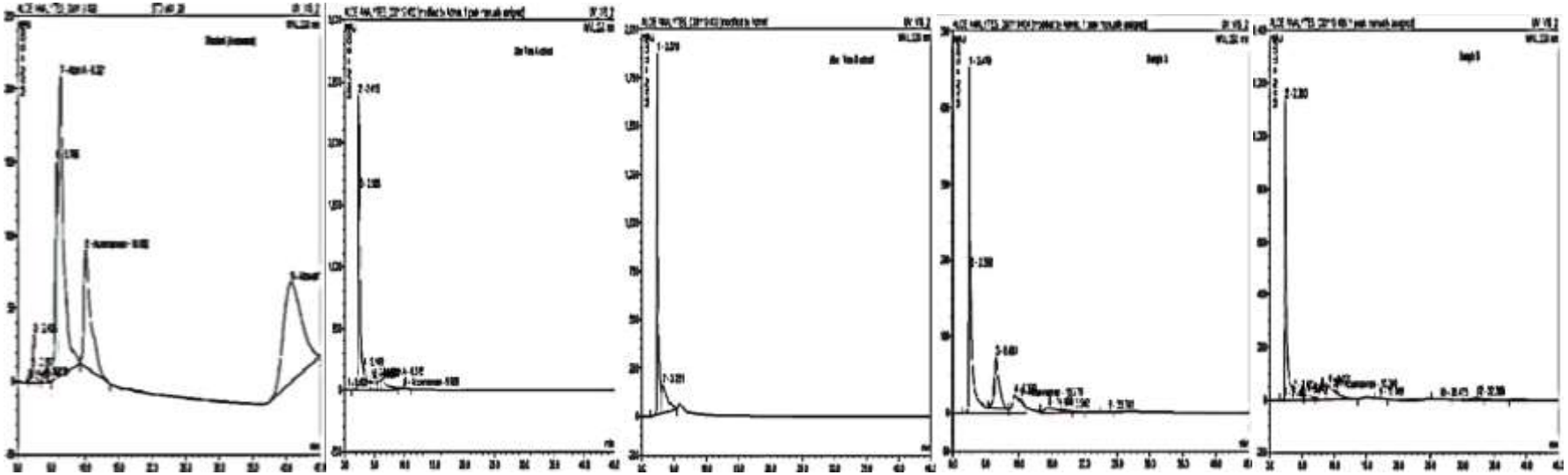


Figure 6. RP-HPLC chromatogram of standard and sample showing presence and absence of Acemannan at 220 nm with retention time of 10.082 min.

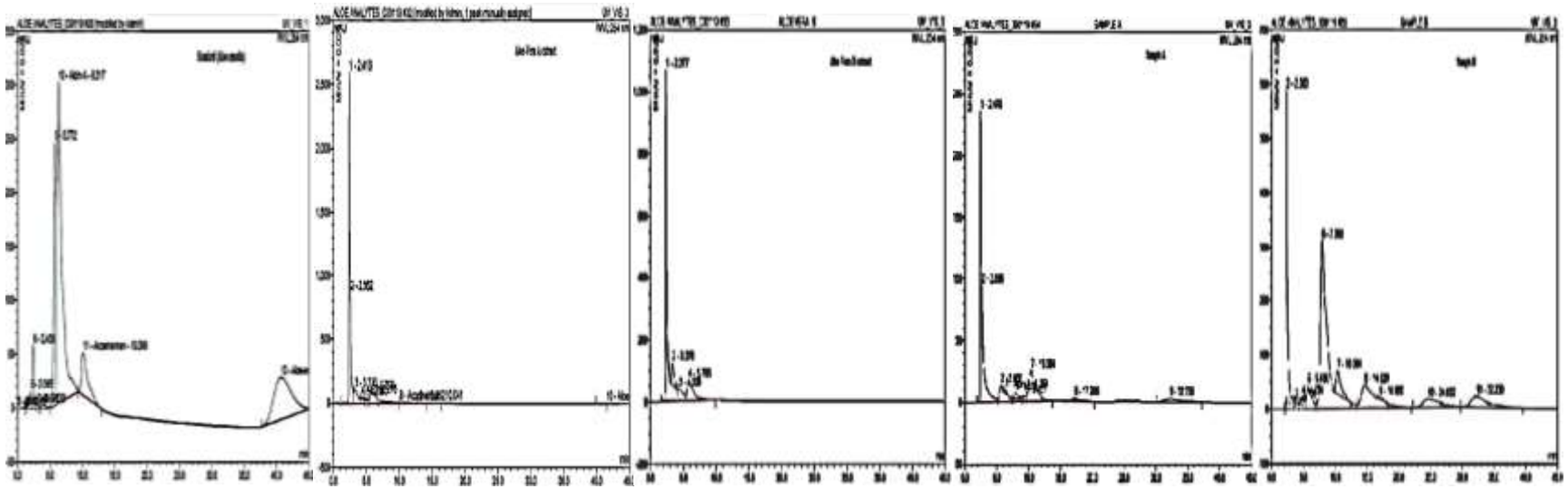


Figure 7. RP-HPLC chromatogram of standard and sample showing presence and absence of *Aloe*-emodin at 254 nm with retention time of 40.711 min

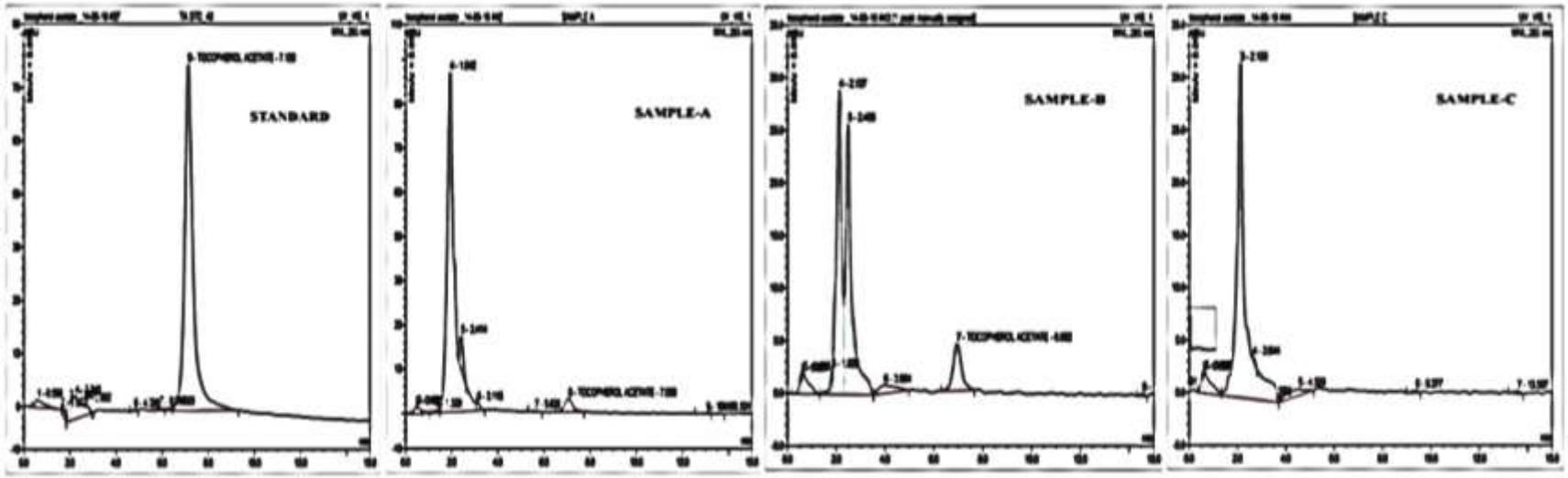


Figure 8. Estimation of tocopherol acetate in the topical formulation sample A at 283 nm with retention time of 7.056 min., sample with retention time of 6.95 min and sample C (nd*).

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Conflict of interest

The authors declare no conflict of interest.

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