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QUALITATIVE AND QUANTITATIVE DETERMINATION OF PHYTOCONSTITUENTS IN MANUALLY PREPARED AVIPATTIKAR CHURNA

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ABSTRACT

Objective: Avipattikar churna a classical Ayurvedic formulation, considered to be a very popular and effective remedy for gastrointestinal problems especially in peptic ulcer and hyper acidity. Well some work has been done its efficacy by in vitro and in vivo experiments. No scientific study was carried on its quantitative analysis. Hence, the present study was to attempt evaluate Avipattikar churna and its ingredients quantifying a mixture of chemical constituents.

Methods: The amount of total phenols calculated as gallic acid was analyzed using Folin–Ciocalteu assay, the amount of total flavanoids calculated as rutin by aluminum chloride assay, total tannins calculated as tannic acid by AOAC method, total reducing sugars by Nelson–Somogyi photometric method and total sugar content by phenol sulphuric acid assay.

Results: In alcoholic extracts of in house Avipattikar churna the TPC, TFC, TTC, TRC and TSC were estimated 12.467±0.273%, 4.734 ± 0.091%, 8.0368 ± 0.138%, 8 %w/w calculated as d-glucose and 50.66667 ± 0.129 respectively.

Conclusion: The result of this study emphasized phenol, flavonoid, tannins, reducing sugars and sugar contents in the different constituents of churna as well as in the major ingredients i.e *Eugenia caryophyllus* and *Ipomoea turpethum*. In this study 1-10 ingredients and clove samples contained highest amount of phenols and flavanoids because of polyphenolic constituents. 1-10 ingredients are

richer in tannins than other samples of the churna. The quantification was done of the individual exploratory samples of aqueous, hydroalcoholic and alcoholic extracts of Avipattikar churna.

Keywords: Avipattikar churna, Spectrophotometry, Total phenolic content, Total flavonoid content, Total tannin content, Total reducing sugars, Total sugar content

INTRODUCTION

For revitalizing the digestive system of humans, the herbal plants are of prime importance. As per global data only 20% of people across the globe are devoid of using herbal plants for their healthy life and treating diseases, rest believe in herbal medications for their wellbeing **[1]**. Avipattikar churna is a well-marketed polyherbal Ayurvedic formulation widely prescribed in the treatment of peptic ulcer and with satisfactory result in patients. It contains around 14 ingredients **(Table 1)**. The poly phenolic compounds like tannins, phenols, flavanoids are of immense importance for the cellular support and shaping out an integral part of polymeric phenolics **[2]**. Present study was performed to explore the preliminary standardization parameters of Avipattikar churna by its different exploratory samples by determining qualitative parameters and quantification of Total phenolic content, total flavanoids content, total tannin content, total sugar content, and total reducing sugar content.

MATERIALS AND METHODS

Chemicals and reagents

Folin-Ciocalteau reagent (1:2) was procured from Qualigens Fine Chemicals, Bombay. Na_2CO_3 (20%w/v), Sodium nitrite (5%), Aluminium chloride (10%), 0.1 N $KMnO_4$, arsenomolybdic acid, alkaline copper reagent, conc. H_2SO_4 and 80% Phenol were procured from S.D. Fines Chemicals Pvt. Ltd., Mumbai. Gallic acid and Rutin was purchased from Yucca enterprises (Mumbai, India). All chemicals and reagents used in the study were of analytical grade. *Zingiber officinale, Embelica officinalis, Terminalia chebula, Piper longum, Embelia ribes, Terminalia bellirica, Piper nigrum,* salt (vidalavana), *Cyperus rotundus*, , *Cinnamomum tamala, Elettaria cardamomum, Syzgium aromaticum, Ipomoea turpethum*, ,*Saccharum officinarum* and Avipattikar churna were procured from an established supplier in Ahmedabad.

Preparation of churna:

Avipattikar churna consists of following fourteen ingredients. All these ingredients were procured from an established supplier and were authenticated at Gujarat University, Ahmedabad. All the ingredients were powdered individually and further they were passed through the sieve (80 #) and blended as per formula **(Table 1)**.

Table 1: Composition of Avipattikar churna

Sr. No	Name of Ingredients	Common name	Quantity
1	*Zingiber officinalis*	Shunthi	1 part
2	*Piper nigrum*	Maricha	1 part
3	*Piper longum*	Pipali	1 part
4	*Terminalia chebula*	Haritaki	1 part
5	*Terminalia bellirica*	Vibhitaki	1 part
6	*Emblica officinalis*	Amalaki	1 part
7	*Cyperus rotundus*	Mustha	1 part
8	*Vida lavana (Salt)*	Salt	1 part
9	*Embelia ribes*	Vidanga	1 part
10	*Eletteria cardamomum*	Ela	1 part
11	*Cinnamomum tamala*	Patra	1 part
12	*Syzygium aromaticum*	Clove	11 parts
13	*Ipomoea turpethum*	Jalap	44 parts
14	Sarkara	Sugar candy	66 parts

The marketed sample of Avipattikar churna was procured from local market, Ahmedabad and the in-house prepared churna were standardised based on their physico-chemical properties

Physicochemical parameters:

Physico-chemical investigations of formulations were carried out including determination of water, hydroalcoholic and alcoholic extractive values of In house Avipattikar churna and marketed Avipattikar churna.

Detemination of pH:

The pH of in house formulations and marketed formulations were determined in 1% w/v and 10% w/v of water soluble portions using standard pH meter at 24°C as mentioned in Ayurvedic pharmacopeia **[3, 4]**.

LOD (Loss on Drying):

Without preliminary drying 10g of each of Avipattikar churna samples were accurately weighted and kept in a tarred petridish for 5 hrs at 105ºC in a hot air oven and percentage was calculated from the difference of initial weight and final weight. Loss on dying value is represented in % w/w.

Phytochemical screening:

Qualitative phytochemical screening was done by performing various chemical tests to confirm the presence of Alkaloids, Carbohydrates, Proteins and amino acids, Glycosides, Flavonoids, Tannins and Phenolics, Steroids, Volatile oils and fats for preliminary identification with freshly prepared reagents **[5-7]**. The preliminary tests were performed in different extracts as per the procedure mentioned in official pharmacopoeia.

Preparation of exploratory samples:

To evaluate the quantitative markers, the in-house churna's components were split into following parts:

a) Powder A: having 1-10 ingredients (those listed in **Table 1**)

b) Powder B: having only Clove

c) Powder C: having only Jalap

d) Powder D (Churna without sugar): having 1-13 ingredients (those listed in **Table 1**)

e) Powder E: in house Avipattikar churna

Estimation of total phenolic contents (TPC):

The total phenolic content in the methanolic extract of different exploratory samples of in house Avipattikar churna were estimated by Folin-Ciocalteau method as exemplified by Singleton & Rossi (1965) **[8, 9]**.

Preparation of standard solutions:

Gallic acid was used as standard for the estimation of TPC. The stock solution of gallic acid (1000µg/mL) was prepared by dissolving 100 mg of gallic acid in 100 mL of methanol. From stock solution further dilutions of standard gallic acid (100, 150, 200, 250, 300, 350, 400, 450 µ/mL) were prepared. Calibration curve was plotted by mixing 1 mL aliquots of different standard solutions of gallic acid, 10 mL of distilled water and 1.5 mL of diluted (1:2) Folin ciocalteu reagent. The mixture was kept aside for 5 min after that 4 mL of 20%w/v Na_2CO_3 solution was added. The final volume was adjusted to 25 mL using methanol and absorbance was measured at 765nm after 30 mins at room temperature against blank (distilled water) in double beam Shimadzu UV-visible spectrophotometer (UV-1800, Singapore).

Preparation of Test extracts:

100 mg each of different exploratory samples (A-E) were macerated with 100 mL of distilled water, alcohol and hydroalcohol (70:30) for 24 hrs. The final volume of the filtrate was adjusted to 100 mL using distilled water, alcohol and hydroalcohol (70:30). To one mL respective extract (100 mg/100 mL), 10 mL of distilled water was added. The same procedure was followed as described for calibration curve. Experiments were measured in triplicates, for the determination of total phenolic compound in individual parts of in-house Avipattikar churna on the basis of a standard curve of gallic acid.

Estimation of total flavanoids (TFC):

Total flavonoid content was calculated as rutin by the method of aluminium chloride **[10]**. For total flavonoid determination, Rutin was used to make the standard calibration curve.

Preparation of standard solution:

10 mg rutin was dissolved in 100 mL volumetric flask using methanol (100 µ/ mL). From the above stock solution pipette out aliquots of 2, 4, 6, 8, 10 mL in to 10 mL volumetric flask and make up the volume with methanol (20-100µg/mL).

Preparation of test sample:

1 g powder of each of the exploratory samples was extracted with 100 mL of distiiled water (aqueous), alcohol and hydroalcohol (70:30). Filtered and made up to 100 mL with aqueous, alcohol and hydroalcohol (70:30) (100mg/mL). In 1mL of standard Rutin solution having concentration 20-100 µg/mL and different test solutions (aliquots extracted in distilled water, alcohol and hydroalcohol (70:30)) of exploratory samples were taken in a 10 mL of volumetric flask and 4 mL of water was added. To this 0.3 mL of 5 % Sodium nitrite, 0.3 mL of 10% Aluminium chloride was added and kept for 5 minutes. 2mL of 1 M Sodium hydroxide was added to each reaction mixture. Immediately the final volume was made upto 10 mL with distilled water. The absorbance of the reaction mixtures was measured against blank at 510 nm on a UV-Vis spectrophotometer. The concentration of total flavonoid content in the exploratory samples was calculated from the calibration plot expressed as mg of rutin equivalent /g of dried plant material. All experiments were executed in triplicate and values were represented in mean ± standard deviation in terms of flavonoid content.

Estimation of total tannin content (TTC):

Total tannins were determined by titrimetric method as per AOAC. 2 gm each of different exploratory samples (A-E) were extracted with 200 mL distilled water, alcohol and hydroalcohol (70:30) by heating for atleast half an hour. Solutions were filtered and adjusted the volume up to 200 mL with distilled water, alcohol and hydroalcohol (70:30) respectively. From the above stock solution 10 mL was taken in a conical flask and 10 mL of indigo carmine solution added and diluted up to 300 mL of distilled water. The individual flask was heated at 70°C and immediately titrated with standardised 0.1 N $KMnO_4$ solution till gave parrot green to golden yellow colour. The same procedure was repeated to determine blank by 10 mL water. The percentage of total tannic acid is calculated by the following factor.

Each mL of 0.1 N $KmnO_4$ = 0.004157 gms of Tannins calculated as Tannic acid

Estimation of total reducing sugar content (TRC):

Total reducing sugars according to Nelson–Somogyi photometric method **[11]** was used to determine total reducing sugars in which glucose is used as standard.

Preparation of calibration curve for d-glucose (Dextrose): 100 mg of dextrose was dissolved in a 100-mL volumetric flask (1 mg / mL). From the above stock solution different aliquots of 0.2, 0.4, 0.6, 0.8 and 1

mL were pipetted out in to 10- mL volumetric flask and diluted with double distilled water to prepare 20, 40,60,80,100 µ/mL concentration of standard solutions.

Preparation of Test extract:

1 g of in house Avipattikar churna is extracted with 10 mL of double distilled water. Filtrate was used to estimate the total reducing sugars. In above standard solutions and test solutions, 1 mL of alkaline copper reagent (Reagent I: 25 g of anhydrous sodium carbonate, 25 g of sodium potassium tartrate, 20 g of sodium bicarbonate and 200 g of anhydrous sodium sulphate were dissolved in 800 ml of water and diluted up to 1 L. Reagent II: 150 g of Copper sulphate and 10 mL conc. H_2SO_4 were dissolved in 800 ml of water and diluted up to 1 L. 25 parts of reagent I and 4 Parts of reagent II were mixed to prepare alkaline copper reagent) was added.

The solutions were boiled for 20 minutes, to this, cooled 1 mL of Nelson reagent (Nelson reagent was prepared by dissolving 250 mg of ammonium molybdate in 45 ml of purified water. 2.1 ml of concentrated sulphuric acid was added. To this solution, 3 g of sodium arsenate was dissolved in 25 ml of purified water, mixed and placed in incubator maintained at 37 º C for 24 hr) and 7 mL of double distilled water were added. The contents of the test tube were mixed and colour intensity was measured at 520 nm. The absorbance was recorded and standard curve of absorbance vs. Concentration were plotted to generate calibration curve.

Estimation of Total sugars by Phenol Sulphuric acid method (TSC): [12, 13]

Preparation of standard solution:

100 mg of glucose is dissolved and diluted with 100 mL of double distilled water (1000 µg/mL) and further diluted with double distilled water to prepare 100 µg/mL. From the above stock solution different mL of aliquots were pipetted out to prepare 20, 40, 60, 80 and 100 µg/mL.

Preparation of Test solutions:

100 mg of Avipattikar churna was extracted with 100 mL of double distilled water. 2 mL of different standard sugar solutions and test solution were pippeted in to volumetric flask. 1 mL of Phenol (80%w/w) and 5mL of conc H_2SO_4 (96%) were added in all the tubes. The mixture was allowed to stand for 10 min. OD was measured at 490 nm by distilled water used as a blank.

RESULTS AND DISCUSSION

Present study was performed to standardize Avipattikar churna through different physico-chemical parameters like LOD, pH, extractive values, phytochemical screening and to quantitatively estimate like total phenolic contents, total flavanoids contents, total tannin contents, total

reducing sugars and total sugar contents in various exploratory samples of churna and in in-house churna, results are represented in the following tables.

Physicochemical parameters

The results of physicochemical parameters of in house Avipattikar churna and marketed churna were compared with standard parameters as per Ayurvedic pharmacopoeia. In house churna showed good results as compared with marketed churna **(Table 2)**.

Phytochemical Screening:

In house churna was showed good results in phyico parameters compared to the marketed formulations. Preliminary phytochemical test results are shown in **Table 3**. It showed presence of carbohydrates, proteins, tannins, flavanoids, phenols, steroids, resin in In house Avipattikar churna.

Estimation of Total Phenolic Content:

Gallic acid was used as a standard and calibration curve was derived as shown in **Figure 1**. The percent of total phenol contents (Gallic acid equivalents) of alcoholic extract of A – E exploratory samples from the absorbance values were calculated as 19.422 ±0.154%, 31.614 ±0.045%, 6.404 ±0.079%, 23.7 ±0.141% and 12.467±0.273% respectively. The percent of total phenol contents (Gallic acid equivalents) of hydroalcoholic extract of A – E exploratory samples from the absorbance values were calculated as 14.816 ±0.0790%, 24.396 ±0.034%, 3.1758 ±0.034%, 11.128 ±0.069% and 5.53±0.027% respectively. The percent of total phenol contents (Gallic acid equivalents) of aqueous extract of A – E exploratory samples from the absorbance values were calculated as 11.049 ±0.107%, 17.624 ±0.391%, 2.650 ±0.026%, 8.503±0.048% and 4.353±0.116% respectively. The experiments were performed in triplicates **(Figure 2)**.

Estimation of Total Flavanoid:

Rutin was used as a standard and calibration curve shown in **Figure 3**. The percent of total flavanoids contents in alcoholic extract samples A-E were calculated as 8.044 ± 0.090, 7.891 ± 0.065, 3.129 ±0.115, 8.788 ±0.122 and 4.735±0.091 respectively. In hydroalcoholic extract samples A-E TFC were calculated as 7.723 ± 0.212, 7.45 ± 0.083, 2.814 ±0.176, and 8.442 ± 0.113 and 4.249 ±0.845 respectively. In aqueous extract samples A-E TFC were calculated as 6.9245 ± 0.097, 6.7106 ± 0.172, 2.451 ±0.069, and 7.6753 ± 0.882 and 3.8226 ±0.180 respectively by aluminium chloride method. The experiments were performed in triplicates **(Figure 4)**. Total phenols and flavanoids of different exploratory sample were represented in **Table 4**.

Total tannin content:

In this study out all the compositions of 1-10 ingredients contained maximum amount of tannin content. So 1-10 ingredients were selected to determine the total tannin content from the samples represented in table 1. 1-10 ingredients sample showed 18.706 ±0.24, 21.200 ±0.24 and 24.664±0.137% of TTC in aqueous, alcoholic and hydroalcoholic extracts respectively by above mentioned AOAC method **(Figure 4)**. Additionally, churna without sugar contained 11.639 ±0.212, 14.549 ±0.24 and 16.905±0.138% of TTC in aqueous, alcoholic and hydroalcoholic extracts respectively. And Avipattikar churna sample E contained 5.958 ±0.138, 8.0368 ±0.138 and 9.976±0.24% of TTC in aqueous, alcoholic and hydroalcoholic extracts respectively. The experiments were performed in triplicates.

Total reducing sugars:

From the calibration curve of glucose, total reducing sugars was estimated 8 %w/w from the regression equation (Y = 0.001x + 0:007, R^2 = 0:997; **Figure 5**) by measuring the OD.

Estimation of Total sugars:

From the calibration curve of glucose, total sugar content was estimated 50.66667 ± 0. 129% w/w from the regression equation (Y = 0.006x – 0.077, R^2 = 0:991; **Figure 6**) by measuring the absorbance.

Table 2: Results of Physicochemical parameters

Parameters	In house Churna	Marketed Churna	As per Ayurvedic Pharmacopoeia
Loss on drying at 105°C	4.4%w/w	6.2%w/w	Not more than 7%
Alcohol Soluble Extractive	23.8%w/w	14.4%w/w	Not less than 20%
Water Soluble extractive	67.4%w/w	65.4%w/w	Not less than 53%
Hydroalcoholic extractive	59.8%w/w	56.8%w/w	-
pH	4.2	3.8	4-6

Table 3: Results of Phytochemical screening

Phytochemicals	Test	Observation
Carbohydrate	Molish's test: 2-3 ml aqueous extracts + α-napthol solution+ concentrated sulphuric acid	Positive
Proteins	Biuret's test: 2-3 mL aqueous extracts + sodium hydroxide (4%) + Copper sulphate (1%)	Positive
Tannins	Ferric chloride test: 2-3 mL aqueous extract + FeCl3 (0.1 %)	Positive
Alkaloids	Dregendroff's test: 2 mL of chloroform extract + dragendroff's test	Negative
Flavonoids	Shinoda Test: Methanolic extract of drug + magnesium ribbon pieces +concentrated hydrochloric acid	Positive
Phenols	Phenol test: 2-3 ml of extracts + glacial acetic acid + Br_2 water	Positive
Saponin glycoside	Foam test: 2-3 mL of aqueous extract + water + shake for 10 mins	Negative
Coumarin or Resin	Fluorescence test: Alcoholic extract + 1 N NaOH solution	Positive
Starch	Iodine test: 2 mL aqueous extract + I_2 reagent	Positive
Steroids	Libermann's burchard test: Chloroform extract of drugs + acetic anhydride + concentrated sulphuric acid	Positive

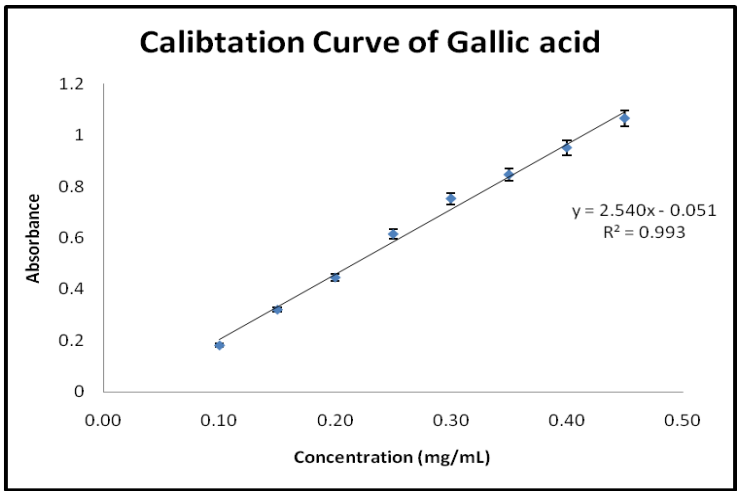

Figure 1: Calibration curve for total phenolic content for standard as Gallic acid

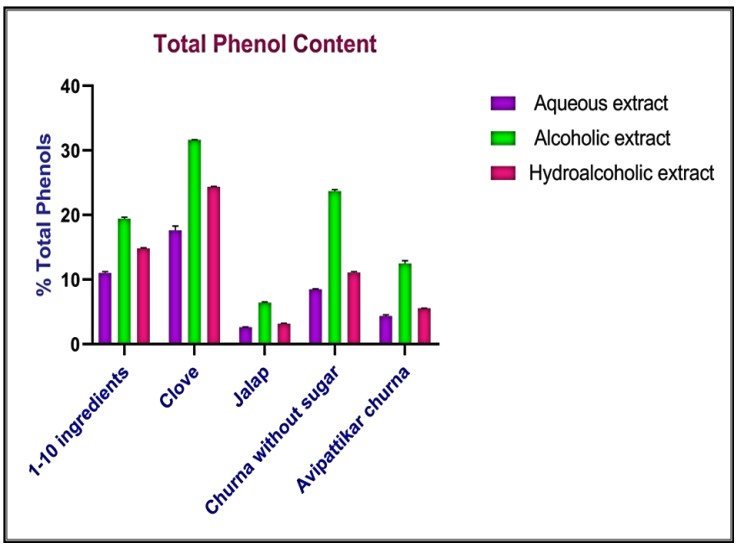

Figure 2: Total Phenolic Content of different exploratory samples

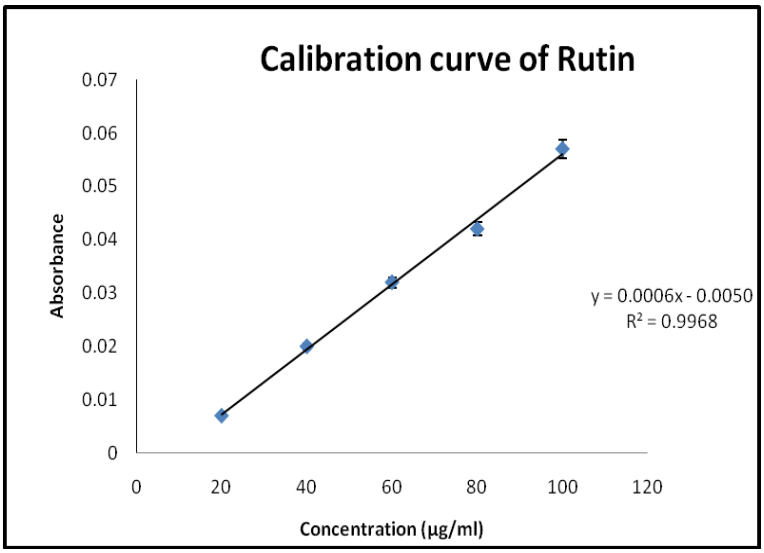

Figure 3: Standard Calibration curve for total flavanoids content for standard as Rutin

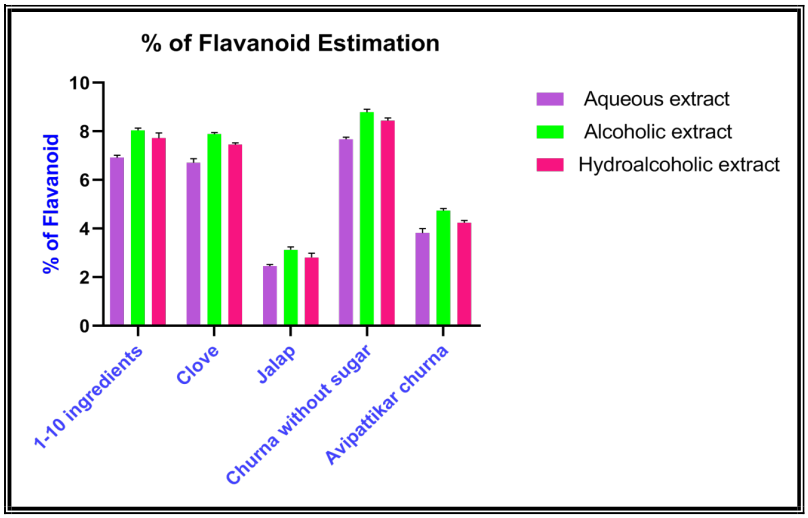

Figure 4: Total flavanoid Content of different exploratory samples

Table 4: Total phenols and flavanoid contents of different exploratory samples

Exploratory Samples	% of Total Phenols (Mean± SEM) (n=3)			% of Flavanoid (Mean± SEM) (n=3)		
	Aqueous extract	Alcoholic extract	Hydro alcoholic extract	Aqueous extract	Alcoholic extract	Hydro alcoholic extract
1-10 ingredients (Sample A)	11.049± 0.107	19.422± 0.154	14.816± 0.079	6.9245± 0.097	8.044± 0.090	7.723± 0.212
Clove (Sample B)	17.624 ± 0.391	31.614± 0.045	24.396± 0.034	6.7106 ± 0.172	7.891± 0.065	7.45 ± 0.083
Jalap (Sample C)	2.650 ± 0.026	6.404± 0.079	3.1758 ± 0.034	2.451 ± 0.069	3.1293 ± 0.115	2.814± 0.176
Avipattikar churna without sugar (Sample D)	8.503± 0.048	23.700 ± 0.141	11.128± 0.069	7.6753 ± 0.882	8.788 ± 0.122	8.442 ± 0.113
Avipattikar Churna (Sample E)	4.353± 0.116	12.467 ± 0.273	5.53± 0.027	3.8226± 0.180	4.734± 0.091	4.249± 0.845

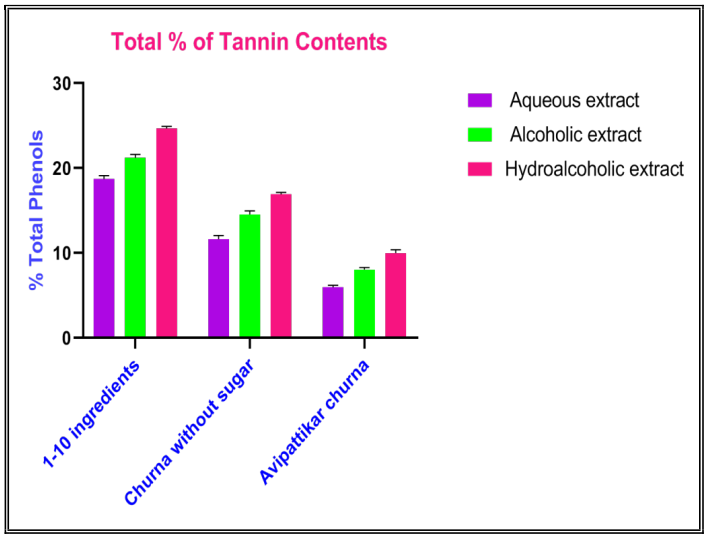

Figure 4: Total tannin content of different exploratory samples

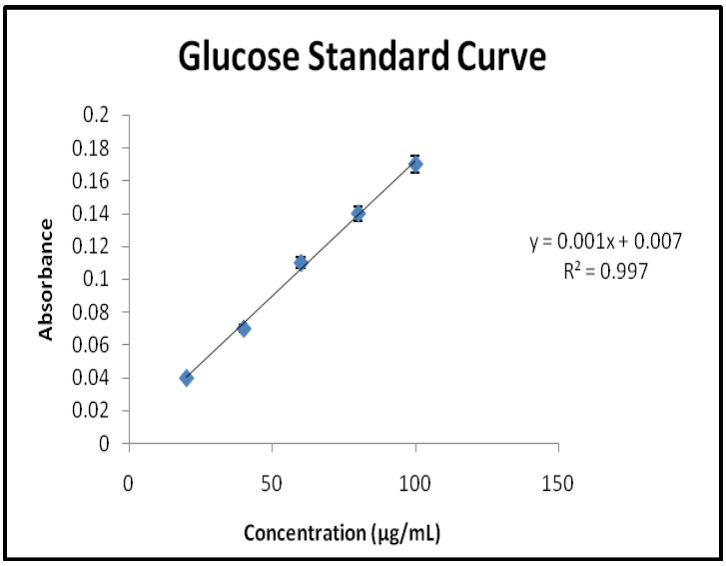

Figure 5: Standard curve of Glucose for total reducing sugars

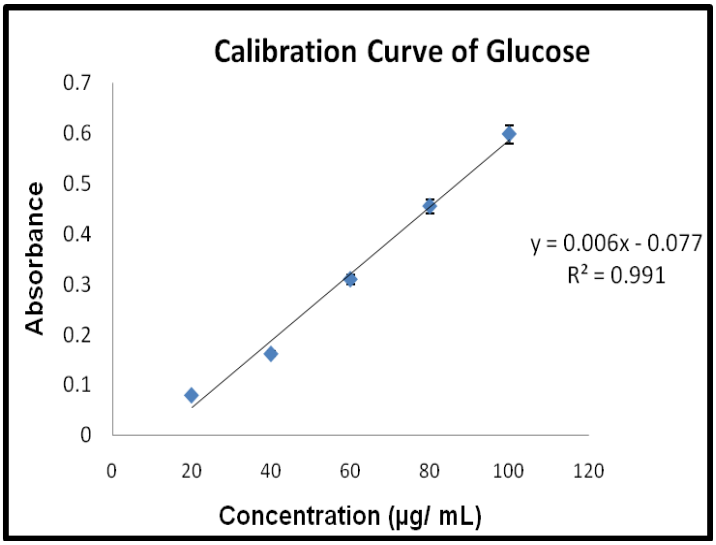

Figure 6: Calibration curve of Total sugars

CONCLUSION:

From extractive value results maximum extractive values were found in hydroalcoholic extract and also in in house Avipattikar churna. So, in house churna was used for standardisation and quantification of various phytochemical parameters.

Phytochemical screening confirmed the presence of phyto-constituents like phenols, flavonoids, sterols, tannins, glycosides, sugars and reducing sugar. In this study the total phenolic and flavanoid contents were reported in exploratory samples of churna. Total phenolic and flavonoid content was found higher in alcoholic extract > hydroalcoholic extract > aqueous extract. The phenol and flavonoid content was found higher in alcoholic extract and lower in aqueous extract of different exploratory

samples. 1-10 ingredients, Clove and Churna without sugar exhibited higher phenolic content in comparison to the other ingredients of churna. Moreover 1-10 ingredients and churna without sugar contained higher total flavanoids. The flavanoids and phenols play an important role in the various antioxidant activities in biological systems. It can be assumed that the classical formulation elixir its activity through various antioxidant mechanism thereby bringing relief in conditions of piles, hyper-acidity and peptic ulcer like other metabolic disorders. Similar mechanisms of these phenolic compounds were also proposed by Sharifi-Rad *et al* **[14]**. In our experiments, alcoholic extracts contained higher amount of flavanoids and phenols indicating that they may be present in polymeric form or in free aglycone form. Total tannin contents were higher in hydroalcoholic extract > alcoholic extract > aqueous extract in different exploratory samples. 1-10 ingredients contained highest amount of tannins because of the equal parts of Amla, Behda, Harde and other 7 tannin rich herbal drugs. The higher amounts of tannins point towards the presence of more alcoholic groups which can be probably due to presence of glycosides. Thus, our study has established a battery of evaluation parameters for Avipattikar churna which helps in the standardization of classical Ayurvedic formulations.

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