Quantification of the important secondary metabolites by HPTLC and antioxidant activity evaluation of *Amartottara Kwatha* (AK) Churna: An Ayurvedic Formulation

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ABSTRACT

There have been no reports of quantification of Catechin in *Amartottara Kwatha* (AK) Churna. Objective of the present work was to prepare AK Churna according to the instructions mentioned in Ayurvedic Pharmacopoeia of India using ingredients as *Zingiber officinale, Tinospora cordifolia, Terminalia chebula*. Aqueous extraction of *Zingiber officinale, Tinospora cordifolia, Terminalia chebula*, AK Churna was done by cold maceration method to perform preliminary phytochemical test and quantitative estimation of phytochemicals. Catechin as important secondary metabolite was estimated in AK Churna using High Performance Thin Layer Chromatography (HPTLC) method and to assess the antioxidant activity of the Aqueous Extract (AE) of AK Churna. AE of AK Churna was applied on silica gel G 60 F254 plate; solvent- chloroform: methanol (6 ml) (1.7:0.3, v/v) and scanned at 254 nm. The AE of AK Churna, *Zingiber officinale, Tinospora cordifolia, Terminalia chebula* was also subjected to antioxidant activity. Findings of preliminary phytochemical test and quantitative estimation of phytochemicals confirmed the presence of alkaloids, tannins, saponins, flavonoids and phenols in the AE of AK Churna, *Zingiber officinale, Tinospora cordifolia* and *Terminalia chebula*. The amount of flavonoids in AE of AK Churna, *Zingiber officinale, Tinospora cordifolia, Terminalia chebula* was found to be 34.60 ± 0.81 mg/100 g, 20.34 ± 0.62 mg/100 g, 19.81 ± 0.11 mg/100 g, 15.35 ± 0.31 mg/100 g respectively. HPTLC analysis resulted well resolved bands for catechin (R_f 0.41). The validated HPTLC method was found suitable for Catechin quantification in AK Churna. The content of flavonoids was found to be maximum in AK Churna. The antioxidant activity of the AE of AK Churna, *Zingiber officinale, Tinospora cordifolia, Terminalia chebula* was assessed. Using the DPPH method, the nitric oxide method, hydrogen peroxide method, and the phosphomolybdate method, antioxidant activity was performed and the results indicated that the AE of AK churna has a significant

level of antioxidant potential in comparison to the AE of *Zingiber officinale, Tinospora cordifolia* and *Terminalia chebula*.

1. INTRODUCTION

Seventy-five to eighty percent of the world's population, primarily in developing countries, continues to rely on traditional medicine for primary healthcare. This is basically due to the widespread perception that traditional remedies are safe, inexpensive, and readily available. The World Health Organization (WHO) has masked that herbal remedies are two to three times more effective than regular drugs. Since the beginning of time, plants have been used for therapeutic purposes and are the origin of modern medicine (Pal and Shukla, 2003). Since the dawn of civilization, the search for cures for chronic diseases are ongoing. Oxidative stress is a major risk factor in the development of many chronic diseases. It is known that free radicals and other reactive oxygen species (ROS) play an important role in the pathophysiology of diseases such as pyrexia, atherosclerosis, Parkinson's disease, Alzheimer's disease, diabetes and asthma. According to certain theories, ROS are responsible for aging. Antioxidants are substances that delay or prevent oxidative damage to a specific molecule. The primary characteristic of an antioxidant is its ability to capture free radicals. By neutralizing free radicals such as peroxide, hydroperoxide, and lipid peroxides, antioxidant substances such as phenolic acids, polyphenols, and flavonoids inhibit the oxidative pathways that result in degenerative diseases. Certain special herbs and Ayurvedic formulations derived from them have been regarded as effective antioxidants since antiquity (Pour et al., 2012). Oxygen is an essential chemical component of aerobic organisms' metabolisms. However, because it may cause unwanted reactions, there is a growing interest in understanding how its ROS operate. ROS include radicals such as the superoxide anion, singlet oxygen, lipid peroxides, and hydroxyl radicals. Oxidative stress is characterized by a rise in ROS production in comparison to their removal by biological systems (antioxidant defences). For quite some time, cancer, diabetes, arthrosclerosis, arthritis, neurological diseases, and premature aging have all been linked to the presence of ROS. The vast majority of naturally occurring antioxidants are derived from plant sources, including fruits, vegetables, herbs and spices (Lourenco et al., 2019).

The most common ROS are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and reactive hydroxyl radicals (OH). Nitric oxide (NO), peroxy nitrite anion (ONOO), nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3) are the other free radicals.

Exogenous ROS are produced by electromagnetic radiation, massive radiation, UV light and tobacco smoke. Endogenous sources of ROS are the mitochondrial electron transport chain and oxidation of fatty acids (Patel et al., 2013). Antioxidants such as L-ascorbic acid and vitamins are essential antioxidants that protect against ROS and these are required to combat ROS. It is important to note that the majority of cancer-preventive agents are derived from plants or chemical mixtures such as phenolic acids and flavonoids that act as antioxidants (Badami and Channabasavara, 2007).

There is substantial evidence that antioxidant rich foods and nutrients, in particular, are essential for prevention of disease. According to the recent findings, antioxidant combinations may be more effective than single antioxidants in the long run. By delaying or preventing the onset of degenerative diseases, antioxidants may be of great importance in enhancing the quality of life. In addition, antioxidants have the potential to significantly reduce the costs associated with health care. Antioxidants have the potential to improve personal satisfaction by delaying the onset of infection degeneration. Recent studies suggest that antioxidants may be effective in both the prevention and treatment of diseases, when administered to the appropriate target (Kurutas, 2016). Literature suggests that some herbs contain a greater concentration of phenolic chemicals than common vegetables. It has been reported that leafy foods have greater antioxidant activity. All phenolic compounds, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids are abundant in medicinal plants with antioxidant properties. An antioxidant's purpose is to trap free radicals by acting as a free radical scavenger and preventing lipid oxidation through chelation and other mechanisms. Antioxidants can mitigate the effects of free radicals and additionally they prevent cell damage. Antioxidants neutralize the damaging effects of free radicals and protect cells from degeneration. Plants are rich in antioxidants, and spices have been used as remedies since ancient times, as evidenced by traditional medicines. Scientists have discovered that regular consumption of antioxidants is necessary to boost the immunity of the body (Wetchakul et al., 2022). In addition to phytochemicals, a number of fungi have recently been found to contain natural, powerful antioxidant compounds that can be used to protect humans from oxidative damage and may help prevent disease (Palma et al., 2022).

AK Churna is an official formulation in the Ayurvedic Pharmacopoeia of India intended for the treatment of fever, cough, cold, sore throat, constipation and loss of appetite. In this study, AK Churna was prepared according to the Ayurvedic Pharmacopoeia of India, and its antioxidant activity was evaluated using *in vitro* models.

For quantitative analysis of active compounds from Ayurvedic formulations, HPTLC is the most precise method for identifying phytochemical markers in plant-based raw materials. HPTLC is a straightforward, sensitive, and precise

technique for fingerprint development and marker-based standardization of plant-derived pharmaceuticals. HPTLC is usually used to identify, test, and measure the purity, stability, solubility, and content uniformity of herbal raw materials and formulations too.

As there is currently no accurate method for quantification of catechin, a method was proposed and validated in accordance with ICH guidelines (ICH 2005). The parameters validated are: Linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), Interday and Intraday Precision and Recovery (%).

2. MATERIALS AND METHODS

Collection and identification of plant materials

The Ayurvedic herbs required for the in-house preparation of AK churna, including Guduchi (*Tinospora cordifolia*) and Ginger (*Zingiber officinale*), were procured from own farm situated at Azamgarh district of India. The dried Haritaki (*Terminalia chebula*) was purchased from Paras Chem Tech Sarkhej in Ahmedabad. The botanical authentication of the specimens (NISCAIR/RHMD/Consult/2019/3525-26-1/2/3) was done from NISCAIR, New Delhi, India. The voucher specimens (COG/PF/016/2019) of *Zingiber officinale* (rhizomes), *Tinospora cordifolia* (stem), and *Terminalia chebula* (fruit) have been deposited in the museum section of NISCAIR, New Delhi, and the Herbarium unit of the Faculty of Pharmacy, IFTM University, Moradabad (UP), India too for future reference. Figures 1 (a), 1 (b), 2 (a), 2 (b), 3 (a), 3 (b), and 4 depict the appearance of *Zingiber officinale*, powdered *Zingiber officinale*, *Tinospora cordifolia*, powdered *Tinospora cordifolia*, *Terminalia chebula*, powdered *Terminalia chebula* and AK Churna respectively.

Fig. 1(a). *Zingiber officinale* **(Sunthi)   Fig. 1 (b). Powdered** *Zingiber officinale* **(Sunthi)**

Fig.2(a). *Tinospora cordifolia* **(Guduchi)**   **Fig. 2(b). Powdered** *Tinospora cordifolia* **(Guduchi)**

Fig. 3(a). *Terminalia chebula* **(Haritaki) Fig.3(b). Powdered** *Terminalia chebula* **(Haritaki)**

Fig. 4. Powdered AK Churna

Preparation of AK Churna

The AK churna was prepared according to the procedure mentioned in Ayurvedic Pharmacopoeia of India. After proper washing and drying, all of the crude drugs of AK churna's crude drugs were ground into powder form. The finely powdered raw materials

were passed through sieve number 60 and combined in the extract proportions of 2:6:4. AK Churna was produced by combining the ingredients in accordance with the proportions specified. The churna was packed in an airtight glass container (Chamundeeswari et al., 2007). In Table 1, the botanical name and amount of each ingredient used to prepare inhouse AK churna are presented.

Table 1: Ingredients of AK Churna

Drug	Botanical Name	Family	Part Used	Quantity
Ginger	*Zingiber officinale*	Zingiberaceae	Rhizome	200 g
Giloy	*Tinospora cordifolia*	Menispermaceae	Stem	600 g
Harad	*Terminalia chebula*	Combretaceae	Fruit	400 g

Preparation of the aqueous extract

Zingiber officinale, *Tinospora cordifolia, Terminalia chebula* and AK Churna were extracted with 500 ml of water using cold maceration method simultaneously. A rotary vacuum evaporator was used to dry the aqueous extracts under reduced pressure. The desiccators were used to preserve the dried extracts, which were then used for further study (Venkateswarlu et al., 2019).

Preliminary Phytochemical Screening

Chemical tests were done for assessing the presence of various secondary metabolites present in AE of AK Churna, *Zingiber officinale*, *Tinospora cordifolia, Terminalia chebula,* (Khandelwal, 2008; Gul et al., 2017).

Quantitative Estimation of Phytochemicals

Fat-free sample preparation- To prepare a defatted sample, 2 g of sample was defatted with 100 ml of diethyl ether using a Soxhlet apparatus for 8 hours.

Total Phenol Determination using Spectrophotometric Method- For phenolic content extraction, 5 ml of the defatted sample was boiled with 50 ml of diethyl ether for 15 minutes. Extract (5 ml) was added to a conical flask (50 ml) along with distilled water (10 ml), ammonium hydroxide (NH_4OH) solution (2 ml), and concentrated amyl alcohol (5 ml). The samples were made to specifications. The mixture was then left to react for 35 minutes. The standard sample was a solution of gallic acid. After color development, the absorbance at 505 nm was measured.

Determination of Alkaloids- The sample (5.0 g) was poured into a 250-ml beaker, and then 10% glacial acetic acid in ethanol (200 ml) was added. Covering the beaker and storing it for 5 hours, the solution was filtered, and the resulting extract was reduced by a quarter in a water bath. Drop by drop, the ammonium hydroxide solution was added to the extract until a precipitate was obtained. The precipitate obtained was washed with a solution of ammonium hydroxide. Finally, the residue of the alkaloid was dried and weighed.

Determination of Flavonoid- At room temperature, 10 g of sample was extracted with 80% aqueous methanol (100 ml). In the study, Whatman filter paper No. 42 (125 mm) was used. The obtained filtrate was poured into a crucible, dried over a water bath, and then weighed.

Saponin Determination- The sample (20 g) was transferred to 20% ethanol (200 ml). The suspension was then heated for four hours at 55 °C in a water bath. The obtained residue was re-extracted with 20% ethanol after filtration (200 ml). The combined extracts were heated to approximately 40 ml in a water bath at 90 °C. Extract was poured into a 250 ml separating funnel, diethyl ether (20 ml) was added, and the mixture was vigorously shaken. The aqueous layer was extracted, whereas the ether layer was left behind. The process of purification was repeated. The extracts (60 ml) were washed twice with 5% sodium chloride aqueous solution (10 ml). The remaining solution was warmed in a water bath. The sample was then dried in an oven to a constant weight. The percent saponin content was determined and presented in Table 5.

Tannins Determination- In a 250 ml volumetric flask, 5g of sample was extracted with distilled water (H_2O) for 5 hr at room temperature. The sample was then filtered. Infusion (25 ml), indigo solution (25 ml), and distilled water (750 ml) were added to a 1 L conical flask. For titration, a 0.1 N potassium permanganate ($KMnO4$) aqueous solution was utilized until the blue colored solution turned green. Afterwards, a few drops were added until the solution became yellow gold in color.

The amount of tannins (T%) in the sample was calculated as follows:

$$T\% = \frac{(v - v_0) \times 0.004157 \times 250 \times 100}{g \times 25}$$

Where v is the volume of 0.1 N $KMnO_4$ solution for titration of sample, ml; v_0 is the volume of 0.1 N $KMnO_4$ solution for titration of blank sample, ml; 0.004157 – tannins equivalent in 1 ml of 0.1 N aqueous solution of $KMnO_4$; g is the mass of sample taken for analysis, g; 250 – volume of volumetric flask, ml.

Chemicals and Reagents
All chemicals and reagents used in the experiment were of analytical grade and obtained from S.D. Fine-chem., Merck, and Fischer Scientific in Mumbai (Krishna et al., 2019). This study made use of a UV-Visible Spectrophotometer (Shimadzu/UV-1800). Ascorbic acid was obtained from Merck, Germany (Annur et al., 2018).

Thin Layer Chromatography of the AK churna extract
20 cm x 20 cm TLC plates with a pre-coated silica gel layer and a glass sheet support were utilized. The thickness of these plates was 0.2 mm (Jamal et al., 2018).
The spotting device utilized was a CAMAG Nanomat 4, which was manufactured in Switzerland by Camag Muttenz. For this procedure, a Hamilton microsyringe of size 10 L was employed. The development chamber was a Camag twin trough chamber with a 20 × 20 cm glass lid. The scan was done with a Camag TLC scanner 4 that was hooked up to Win CATS software (Tuzimski, 2010).
In order to select the solvent system for AK churna extract, a number of attempts were made to improve resolution and determine the maximum number of spots. A good solvent system with the ratio of chloroform to methanol was 1.7 to 0.3 was selected (Kumari et al., 2017).

Quantification of Catechin using HPTLC

Sample solution

For HPTLC, 1 g of aqueous extract of inhouse prepared AK Churna was mixed with water to make the sample solution (Halstead et al., 2007).

Preparation of standard solution of Catechin

In a volumetric flask, 1 mg of precisely weighed catechin was dissolved in water, and the volume of the solution was brought to 100 ml using water. This resulted in stock solutions of catechin with a 10 g/ml concentration. Standard solutions with concentrations of 2, 4, 6, 8 and 10 ml were transferred to 10 ml volumetric flasks and each vial was filled with water to get standard solutions with concentrations of 2, 4, 6, 8 and 10 mg/ml of catechin, respectively, in each vial (Gottumukkala et al., 2014). The structure of Catechin is presented in Figure 5.
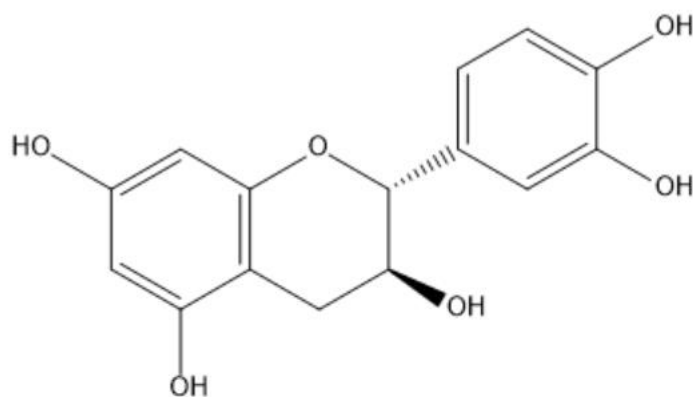

Fig. 5. Catechin

Preparation of calibration curve of catechin

Standard solutions of catechin were applied to a TLC plate with the parameters (band width: 5 mm; distance between the tracks: 10 mm) set as follows: 10 ng/spot, 20 ng/spot, 30 ng/spot, 40 ng/spot, and 50 ng/spot. We used a solvent system chloroform: methanol (6 ml) (1.7:0.3, v/v) solvent system in a CAMAG twin trough chamber (20 x 20 cm) at 25 ± 2 °C and 45 percent relative humidity. Anisaldehyde–sulfuric acid reagent was used to derivatize and heat the plates at 110 °C for 3 m before a densitometric scan was performed using UV light. The resolved peak area was measured (Khan et al., 2011).

To find out the concentration of catechin, calibration curve was drawn and an equation was made with peak areas and catechin concentrations as inputs (Chignola et al., 2022).

Validation of the method

Using the ICH guidelines, the analytical method was validated in order to find out precision, repeatability, accuracy, LOD, LOQ etc (Choudhary et al., 2020).

Precision

Repeated scanning (n=6) of the same spot of catechin (20 ng/spot) was used for the precision study, and the results is presented as a relative standard deviation (% R.S.D.) (Chaudhary et al., 2022).

Inter-day and intra-day variation

By analysing aliquots of standard solutions of 10, 20, and 30 ng/spot of catechin on the same day (intra-day precision) and on different days (inter-day precision), the method's variability was calculated, and the results are presented as a percentage of RSD (Jain et al., 2021).

Limit of Detection (LOD) and Limit of Quantification (LOQ)

For the purpose of determining the LOD and LOQ, various concentrations of standard solutions of catechin were utilized, and water was used as a blank. The results of these experiments were based on the root of the signal to noise ratio. The LOQ was determined to be present at a S/N of 8 ng, while the LOD was found to be present at 4 ng (Saadati et al., 2013).

Repeatability

The repeatability of the method that was made was tested by putting 20 ng/spot of catechin on a TLC plate and analysing each spot individually (n = 6). The results are presented as RSD (Hussain et al., 2014).

Specificity

Analysis of the standard compound and the samples were included in the specificity study. The Rf and spectra of the band were compared to those of the standard, which allowed for the confirmation of the bands representing catechin in the sample solutions (Dharmender et al., 2010).

Table 2
TLC fingerprinting profile of AK churna extract (sample solution and standard solution; solvent system) under UV 366 nm (Jain et al., 2021).

S. No	Peak No	R_f Value	Colour of Band
1.	1.	0.31	Brown
2.	2.	0.34	Brown
3.	3.	0.40	Brown
4.	4.	0.41 (Catechin)	Brown
5.	5.	0.48	Light Brown
6.	6.	0.50	Light Brown
7.	7.	0.59	Yellow

8.	8.	0.62	Yellow
9.	9.	0.77	Yellow
10.	10.	0.81	Yellow

Table 3

Method validation parameters for the quantification of catechin by the proposed TLC densitometric method.

S. No	Parameter	Value
1.	Precision (% RSD, n = 6)	0.98
2.	Repeatability (% CV, n = 6)	1.66
3.	Accuracy (mean recovery)	99.62
4.	Limit of Detection (LOD) (ng)	4 ng
5.	Limit of Quantification (LOQ) (ng)	8 ng
6.	Specificity	Specific
7.	Linearity (correlation coefficient)	0.998
8.	Range (ng/spot)	10-50

Recovery

An evaluation of the reliability of the method was carried out by carrying out recovery studies at three distinct levels (50 percent, 100 percent, and 125 percent addition of Catechin). Calculations were done to determine the percent recoveries and the mean recovery.

Quantification of marker compound in the sample

On a TLC plate, 5 μl of sample solution of an aqueous extract was put down twice. The plates were scanned and developed. With the help of the calibration curve, the areas of the peaks were written down, and the amount of catechin was figured out (Hamidi et al., 2017).
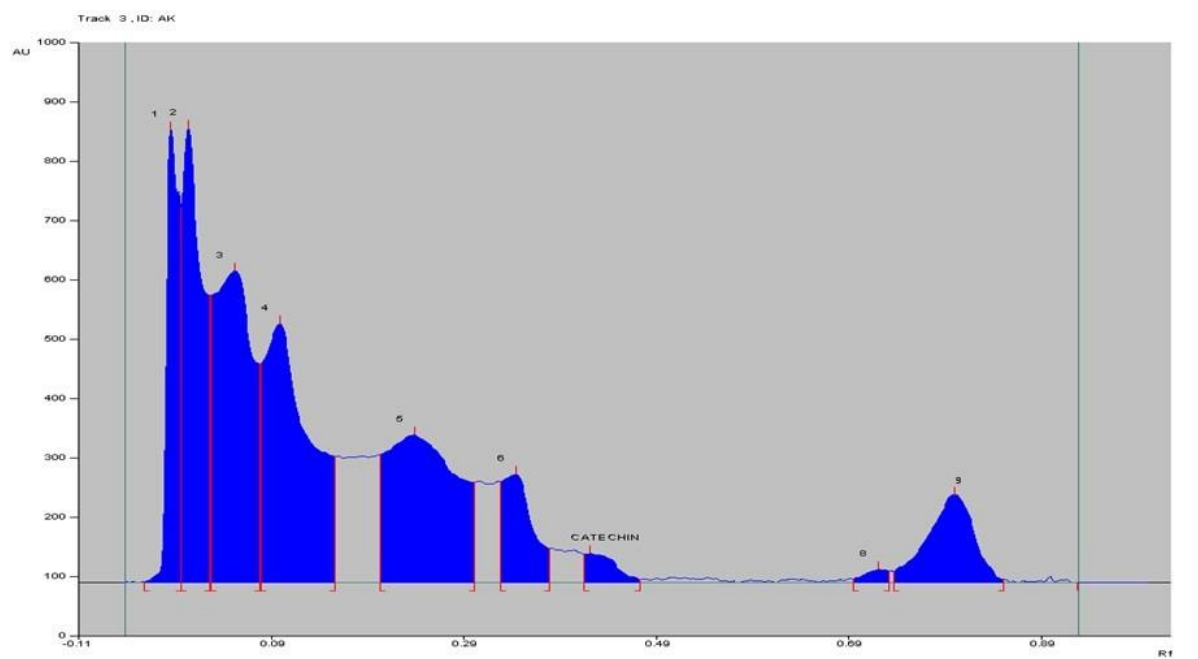
Fig 1 TLC densitometric scan at 366 nm of   test sample solution
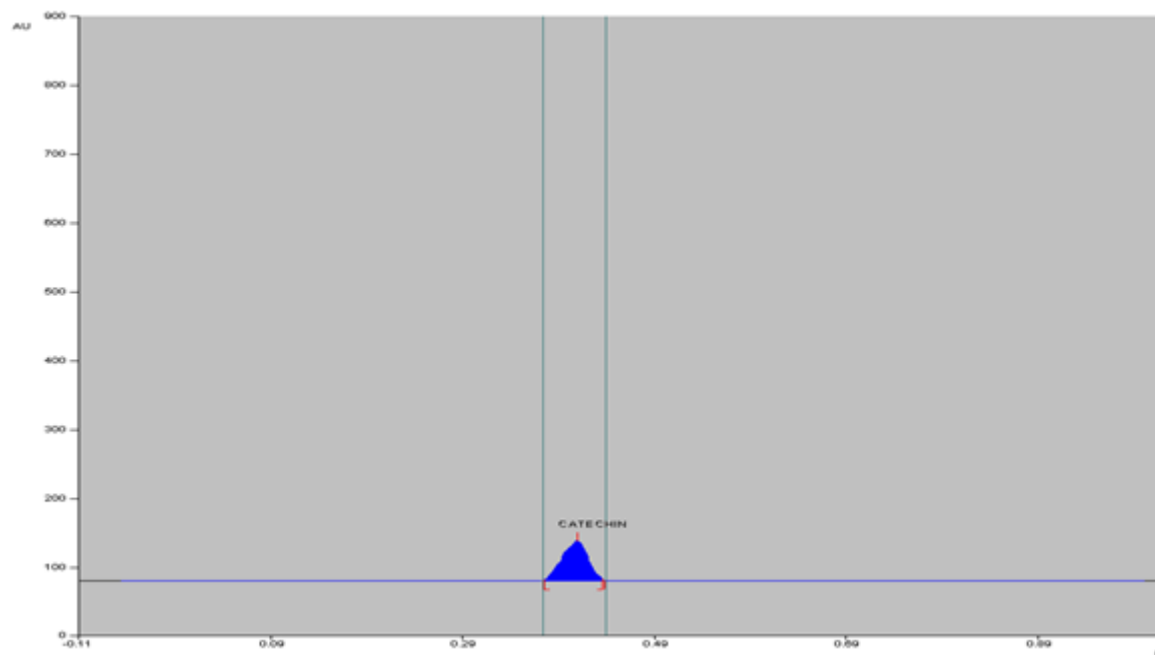

Fig. 2. TLC densitometric scan at 366 nm of catechin standard solution

The Content of catechin quantified using HPTLC densitometric method was found to be 1.25 ± 0.04 µg/g of Ak Churna.

Total Phenol Content

In separate conical flasks, 50 ml of water and 5 gm of dry powdered AK churna were mixed and macerated to get the extract. The mixture was stirred on a magnetic stirrer for 1 hr at 37°C, room temperature. A filter paper was used to filter the content, and the amount of the extract was recorded. The following tests were run using the extracts mentioned above. The Folin-Ciocalteu method was used to calculate the total phenolic content of the extract. 200 mL of crude extracts (1 mg/ml) were diluted to a volume of 3 ml with distilled water, mixed well with 0.5 ml of Folin-Ciocalteu reagent for 3 m, and then 2 ml of 20% (w/v) sodium carbonate were added. At 650 nm, absorbance was measured after the mixture had stood for a further 60 m at 40 °C in the dark. The calibration curve was used to calculate the total phenolic content, and the results are shown as mg of gallic acid equivalent per g of dry weight (Baba and Malik, 2015).

Evaluation of Antioxidant Activity

1, 1- diphenyl -2- picrylhydrazyl (DPPH) radical scavenging assay

The potential of aqueous extract of AK Churna to scavenge DPPH free radicals was calculated. In order to create the aqueous solution of DPPH (0.1 mm) in ethanol, 1.9 mg of DPPH was dissolved in 100 ml of ethanol. For 30 m, the solution was kept in the dark to allow the reaction to finish. Different concentrations of AK churna extracts (50, 100, 1500, 200, and 250 mg/ml) were mixed with 1 ml of DPPH solution, and the mixture was left to stand at room temperature for 30 m.

The mixture was measured using spectrophotometry at 517 nm with a Shimadzu UV-1800 UV-VIS spectrophotometer.

The free radical scavenging activity was determined by using the following formula:-

$$\% \text{ DPPH radical scavenging activity} = (A_0 - A_1)/A_0 \times 100$$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of the sample. Ascorbic acid was used as standard. From the absorbance obtained, percentage inhibition and regression curves were made, and the linear equation was applied to calculate the IC_{50} value (Behera, 2018; Shabi et al., 2017).

Hydrogen Peroxide Radical Scavenging (H_2O_2) Assay

According to the Indian Pharmacopoeia 1996, 0.2 M potassium dihydrogen phosphate and 0.2 M sodium hydroxide solutions were created. To make phosphate buffer, a 200 ml volumetric flask was filled with 50 ml of potassium dihydrogen phosphate solution and followed by 39.1 ml of 0.2 M sodium hydroxide solution, and 200 ml of distilled water (pH-7.4) was adjusted. In order to generate the free radicals, 50 ml of phosphate buffer solution was mixed with an equivalent volume of hydrogen peroxide. The mixture was then set aside at room temperature for 5 m to complete

the reaction. The absorbance was measured at 230 nm using spectrophotometer (UV-1800, UV-VIS spectrophotometer, Shimadzu) against a blank solution containing phosphate buffer solution only. After the addition aqueous extract of the AK churna (1 ml) in distilled water was added to 0.6 ml of hydrogen peroxide solution. Concentrations of between 50 and 250 mg/ml were chosen for the extract. Ascorbic acid was used as a standard. The tests were carried out three times.

The percentage of scavenging of H_2O_2 of extract was measured using the following equation:

$$\% \text{ NO radical scavenging activity} = (A_0 - A_1)/A_0 \times 100$$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of the sample (Shabi et al., 2017).

Nitric Oxide Scavenging Assay

Nitric oxide scavenging method was employed using Griess reagent. In the present investigation, Griess reagent was prepared by utilizing naphthyl ethylene diamine dihydrochloride (0.1% w/v). The combination (3 ml) containing 2 ml of 10 mM sodium nitroprusside, 0.5 ml phosphate buffered saline and aqueous extract of AK (50-250 µg/ml) were subjected to the temperature at 25°C for 150 m. After incubation, 0.5 ml of the reaction mixture was mixed with 1 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 m for the completion of the reaction of diazotization. After this, 1 ml of the naphthyl ethylene diamine

dihydrochloride was mixed and was allowed to stand for 30 m at 25°C. The concentration of nitrite was assayed at 546 nm and the same was calculated with the control absorbance of the standard nitrite solution (without extracts or standards). The buffer solution was used as blank solution and Ascorbic acid was taken as standard solution.

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The percentage inhibition was calculated using the formula:

$$\% \text{ Scavenging activity} = [(A_{control} - A_{test} \text{ or } A_{std})/A_{control}] * 100$$

Where $A_{control}$ is the absorbance of control and A_{test} or A_{std} is the absorbance of test or standard, respectively (Patel et al., 2010; Sharma et al., 2015).

Phosphomolybdate Assay

Phosphomolybdate assay was used for antioxidant evaluation of AK Churna extract. Phosphomolybdate reagent was prepared by mixing 100 ml of 28 mM sodium phosphate, 100 ml of 0.6M sulphuric acid and 100 ml of 4 mM ammonium molybdate solutions. To 3 ml of phosphomolybdate reagent, 300 µl of different concentrations (50-250 µg/ml) of AK Churna extract solution were added and incubated in the dark for 90 m at 95°. The absorbance was measured spectrophotometrically at 765 nm. Ascorbic acid was used as standard solution (Krishna et al., 2019).

Statistical Analysis

Data were expressed as mean ± SEM of triplicate determinations. Linear

regression analysis was used to calculate the IC50 values.

3. RESULT AND DISCUSSION

Preliminary Phytochemical Screening

The preliminary phytochemical observations revealed the presence of tannins, alkaloids, saponins, flavonoids in AE. The findings are presented in Table 4.

Table 4 Phytochemical screening of AE of AK Churna

S.No.	Phytoconstituents	AE of AK Churna	AE of *Zingiber officinale*	AE of *Tinospora cordifolia*	AE of *Terminalia chebula*
1.	Alkaloids	+	+	+	+
2.	Tannins	+	+	+	+
3.	Glycosides	-	-	-	-
4.	Saponins	+	+	+	+
5.	Steroids	-	-	-	-
6.	Flavonoids	+	+	+	+
7.	Terpenes	-	-	-	-
8.	Carbohydrates	-	-	-	-
9.	Phenols	+	+	+	+

+ = Present; - = Absent

Quantitative Estimation of Phytochemicals

The amount of alkaloids, tannins, saponins, flavonoids, phenols present in AE of AK Churna are presented in Table 5.

Table 5 Quantitative estimation of phytochemicals

Sample	Tannins mg/100g	Alkaloids mg/100g	Phenols mg/100g	Flavonoids mg/100g	Saponins mg/100g
AE of AK Churna	9.45±0.10	7.82 ± 0.68	320.19 ± 4.08	34.60 ± 0.81	6.33 ± 0.58
AE of *Zingiber officinale*	8.23±0.08	6.31 ± 0.17	237.64 ± 1.88	20.34 ± 0.62	5.12 ± 0.34
AE of *Tinospora cordifolia*	8.10±0.10	5.24 ± 0.52	286.16 ±1.39	19.81 ± 0.11	4.88 ± 0.63
AE of *Terminalia chebula*	7.45±0.07	5.22 ± 0.41	488.76 ±1.07	15.35 ± 0.31	3.55 ± 0.57

Data is presented as mean ± SEM

Fingerprinting by HPTLC

In order to ensure the quality of the any ayurvedic product, chromatographic fingerprint analysis is used to determine the number of active ingredients from plant species that have

traditionally been used for therapeutic purposes. A variety of chromatographic techniques are utilized for identification and quantification of ingredients in ayurvedic formulations. With the assistance of the developed fingerprint pattern, it is possible to ascertain both the ratio of all analysable markers and the fingerprint pattern of the various markers of interest. High-pressure thin-layer chromatography (HPTLC) is one of the good analytical techniques used for analysis of Ayurvedic sample.

HPTLC is still considered one of the most efficient methods for determining the quality and quantity of ayurvedic formulations. The HPTLC technique is commonly used for the development of chromatographic fingerprints for botanicals and other herbals.

Table 6 Intra-day and inter-day precision of catechin

Marker	Concentration (ng/spot)	Intra-day precision	Inter-day precision
Catechin	10	0.44	0.25
	20	0.33	0.42
	30	0.38	0.50

% R.S.D.; mean (n= 3).

In case intra-day and inter-day precision study, the findings are less than 2 in each case which suggest the method in precise for quantification of catechin.

Table 7 Results of recovery of catechin with standard addition at 50%, 100% and 125% addition by the developed HPTLC method.

Marker Compound	Marker quantity present (ng)	Quantity of marker added (ng)	Quantity of marker found (ng) mean ± SEM	Recovery (%) mean ± SEM	Average recovery (%)
Catechin	50	25	24.31±0.18	99.52	
	50	50	49.11±0.81	98.22	**98.91**
	50	62.5	60.88±0.32	99.01	

The recovery study is done to find out the level of accuracy of proposed method. In present study, the recovery is 98.1 % which is indicative of good accuracy of proposed method.
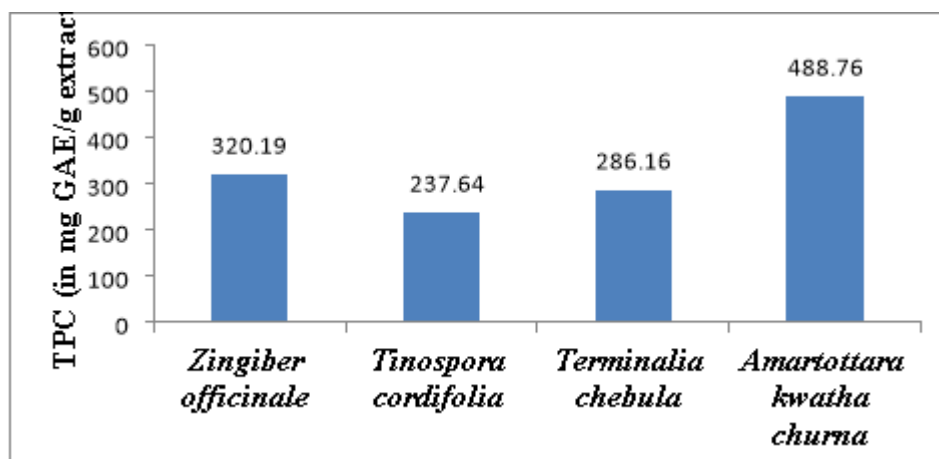
Total Phenolic Content

The total phenolic concentration of different AE of *Zingiber officinale, Tinospora cordifolia, Terminalia chebula* and AK churna is presented in figure 5. The total phenolic compounds were quantified by Folin-Ciocaltue reagent method. This assay is based on the reduction of Folin's reagent by the phenolic compounds. Since phenols dissociate into phenolate anion and protons under an alkaline pH condition, phenolate anion can reduce Mo (VI), a significant component of Folin's reagent, by a single electron transfer. Due to this reduction, a blue coloured complex ($PMoW_{11}O_{40}$) is formed with an absorption max at 750 nm. Among

the studied extracts, the aqueous extract of AK churna exhibited highest level of total phenolic concentration (488.76 mg GAE/L) whereas of *Zingiber officinale, Tinospora cordifolia* and *Terminalia chebula* showed phenolic concentration at 320.19, 237.64 and 286.16 (mg GAE/L)) respectively. Findings of total phenolic content are presented in Table 7 and Figure 6.

Table 7: Total phenolic content of AE of AK churna, *Zingiber officinale, Tinospora cordifolia, Terminalia chebula*

Aqueous Extract (AE)	Total Phenolic Content (mg equivalent/g of extract)
AK churna	320.19 ± 4.08
Zingiber officinale	237.64 ± 1.88
Tinospora cordifolia	286.16 ±1.39
Terminalia chebula	488.76 ±1.07

Fig. 6. Total phenolic content (TPC) of AE of *Zingiber officinale, Tinospora cordifolia, Terminalia chebula* and *AK* churna

Antioxidant activity of *Amrtottara Kwatha* churna

DPPH radical scavenging activity

The DPPH radical scavenging activity of different AE of *Zingiber officinale, Tinospora cordifolia, Terminalia chebula* and AK churna were performed and presented in the Figure 7. The DPPH method has been employed to evaluate the antioxidant potential of a variety of plant extracts. A free radical called DPPH (2,2,2,2-diphenyl-1-picrylhydrazyl) exhibits hydrogen acceptor activity toward antioxidants and has a dark purple color with the highest retention at 515 nm. Antioxidants can diminish DPPH by introducing hydrogen into its reduced structure (DPPH-H), which causes the ingested material to disappear at 515 nm. The reaction between phytochemicals and DPPH, which

demonstrates the antioxidant power, may be the source of the drop in receptiveness at 515 nm. In the present work, the DPPH method was used to measure antioxidant activity by immediately monitoring color shifts from purple to yellow. The AE of *Zingiber officinale*, *Tinospora cordifolia*, *Terminalia chebula* and AK churna exhibited a maximum DPPH scavenging activity of 72.54 %, 74.44 %, 69.68 % and 89.44 % at 250 μg/ml whereas for ascorbic acid (standard) was found to be 92.53 % at 250 μg/ml.

The IC_{50} values of aqueous extract of *Zingiber officinale*, *Tinospora cordifolia*, *Terminalia chebula* and AK churna were found to be 281.50 (μg/ml), 251.35 (μg/ml), 282.18 (μg/ml) and 165.28 (μg/ml) respectively. Whereas The IC_{50} values of Ascorbic acid (standard) was found to be 163.49 (μg/ml).

Findings of DPPH radical scavenging activity of *Zingiber officinale*, *Tinospora cordifolia*, *Terminalia chebula* and *Amrtottara Kwatha* churna are presented in Table 8 and Figure 6.

Table 8: DPPH radical scavenging activity of Zingiber *officinale*, *Tinospora cordifolia*, *Terminalia chebula* and *Amrtottara Kwatha* churna

Concentration (μg/ml)	% Inhibition				
	Zingiber officinale	*Tinospora cordifolia*	*Terminalia chebula*	*Amrtottara Kwatha*	Ascorbic Acid
0	0	0	0	0	0
50	35.35	41.05	39.23	41.25	49.62
100	41.23	46.32	43.15	54.32	62.42
150	53.5	55.32	49.23	66.23	70.39
200	64.32	65.83	60.25	73.52	78.32
250	72.54	74.44	69.68	89.44	92.53
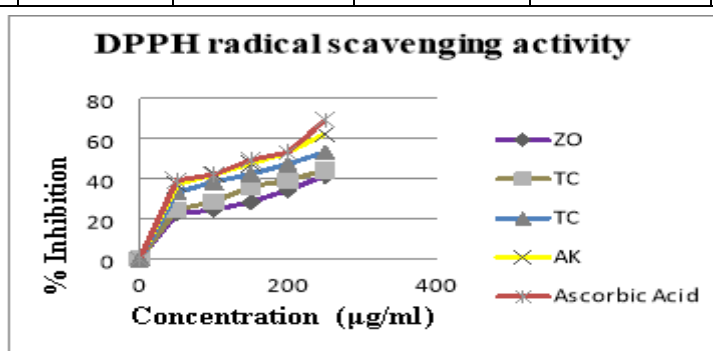
Fig. 7. DPPH radical scavenging activity of *Zingiber officinale* (ZO), *Tinospora cordifolia* (TC)*, Terminalia chebula* (TC) and *AK* (AK) churna

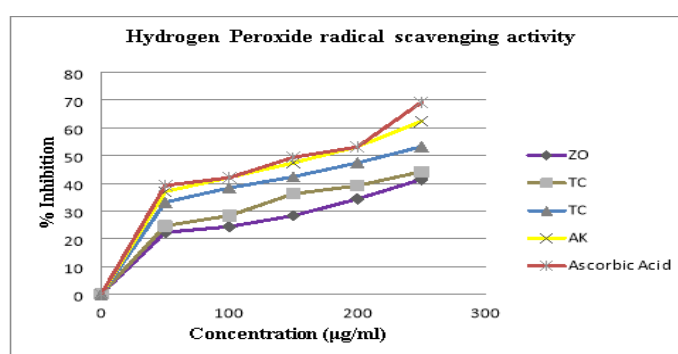**Hydrogen peroxide radical scavenging (H_2O_2) assay**

Hydrogen peroxide itself isn't exceptionally responsive; however, it can in some cases be poisonous to cells since it might bring about hydroxyl revolution in the cells. Accordingly, eliminating hydrogen peroxide is vital for the assurance of cell framework. The hydrogen

peroxide can deteriorate into the water by accepting two electrons and protons. The degree of hydrogen peroxide in cushion arrangement can be recognized spectrometrically at 230 nm. Figure 8 uncovers the hydrogen peroxide restraint action of *Zingiber officinale*, *Tinospora cordifolia, Terminalia chebula* and AK churna. The AE of *Zingiber officinale*, *Tinospora cordifolia, Terminalia chebula* and AK churna exhibited H_2O_2 scavenging activity of 68.74 %, 69.20 %, 68.23% and 82.31 % at 250 µg/ml whereas for ascorbic acid (standard) was found to be 90.31 % at 250 µg/ml. The IC_{50} values of AE of *Zingiber officinale*, *Tinospora cordifolia, Terminalia chebula* and AK churna were found to be 296.3 (µg/ml), 283 (µg/ml), 224.7 (µg/ml) and 174.21 (µg/ml) respectively. Whereas The IC_{50} value of Ascorbic acid (standard) was found to be 156.31 (µg/ml). On comparison, it was found that AK churna exhibit maximum antioxidant activity at 82.31 %.

Findings of Hydrogen peroxide radical scavenging activity of Zingiber *officinale*, *Tinospora cordifolia, Terminalia chebula* and *Amrtottara Kwatha* churna are presented in Table 9 and Figure 8.

Table 9: Hydrogen peroxide radical scavenging activity of Zingiber *officinale*, *Tinospora cordifolia, Terminalia chebula* and *AK* churna

Concentration (µg/ml)	% Inhibition				
	Zingiber officinale	*Tinospora cordifolia*	*Terminalia chebula*	*Amartottara Kwatha*	Ascorbic Acid
0	0	0	0	0	0
50	20.31	26.32	38.5	52.13	56.21
100	32.21	38.4	43.2	62.36	65.32
150	41.41	46.5	51.2	69.21	72.32
	53.44	52.1	61.33	73.21	81.23
250	68.74	69.2	68.23	82.31	90.31

Fig. 8. Hydrogen Peroxide radical scavenging activity of *Zingiber officinale* (ZO), *Tinospora cordifolia* (TC)*, Terminalia chebula* (TC) and *Amartottara Kwatha* (AK) churna

Nitric Oxide Scavenging Activity

Free radical nitric oxide is formed when sodium nitroprusside reacts with oxygen to produce nitrite. The extracts' ability to directly interact with oxygen, nitric oxide, and other nitrogen

molecules allowed them to directly block the generation of nitrite, which was used to measure the antioxidant activity. The current review demonstrates that the rising centralization of the concentrate have a greatest inhibitory movement against the nitric oxide. The nitric oxide scavenging action of AE of *Zingiber officinale*, *Tinospora cordifolia*, *Terminalia chebula* and AK churna are displayed in Fig 9. The AE of *Zingiber officinale*, *Tinospora cordifolia, Terminalia chebula* and and AK churna exhibited nitric oxide scavenging activity 61.22 %, 59.20 %, 62.23 % and 79.31 % at 250 µg/ml whereas for ascorbic acid (standard) was found to be 83.31%.

The IC_{50} values of AE of *Zingiber officinale*, *Tinospora cordifolia, Terminalia chebula* and AK churna were found to be 303.7 (µg/ml), 281.2 (µg/ml), 223.81 (µg/ml) and 173.35 (µg/ml) respectively. Whereas The IC_{50} values of Ascorbic acid (standard) was found to be 148.7 (µg/ml). On comparison among *Zingiber officinale*, *Tinospora cordifolia, Terminalia chebula and* AK formulation, it was found that AK churna exhibit maximum antioxidant activity79.31 % but it is lower from Ascorbic acid 83.31 %.

Findings of Nitric Oxide scavenging activity of Zingiber *officinale*, *Tinospora cordifolia, Terminalia chebula* and *Amrtottara Kwatha* churna are presented in Table 10 and Figure 9.

Table 10: Nitric Oxide scavenging activity of *Zingiber officinale*, *Tinospora cordifolia, Terminalia chebula* and *Amrtottara Kwatha* churna

Concentration (µg/ml)	% Inhibition				
	Zingiber officinale	*Tinospora cordifolia*	*Terminalia chebula*	*Amartottara Kwatha*	Ascorbic Acid
0	0	0	0	0	0
50	13.2	11.2	16.32	42.3	49.22
100	22.31	25.34	43.12	51.36	56.32
150	32.22	33.45	51.56	60.21	62.32
200	45.78	42.1	59.64	67.24	71.23
250	61.22	59.2	63.23	79.31	83.31
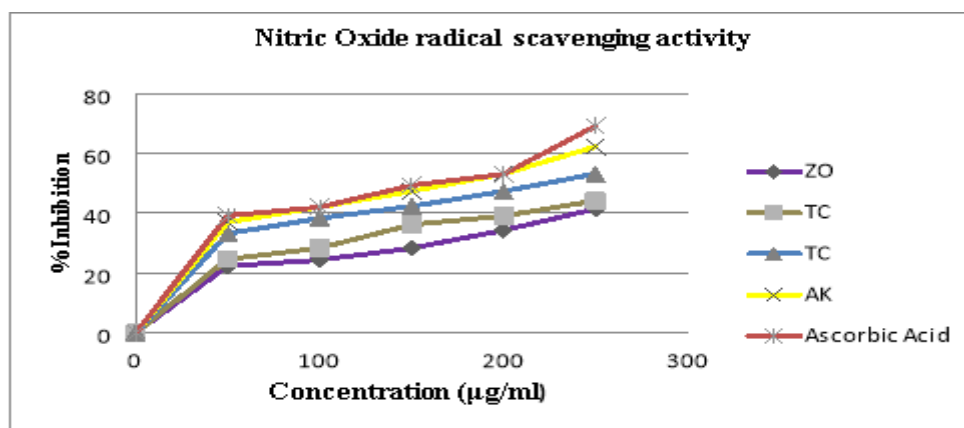
Fig. 9. Nitric Oxide radical scavenging activity of *Zingiber officinale* (ZO), *Tinospora cordifolia* (TC)*, Terminalia chebula* (TC) and *Amartottara Kwatha* (AK) churna
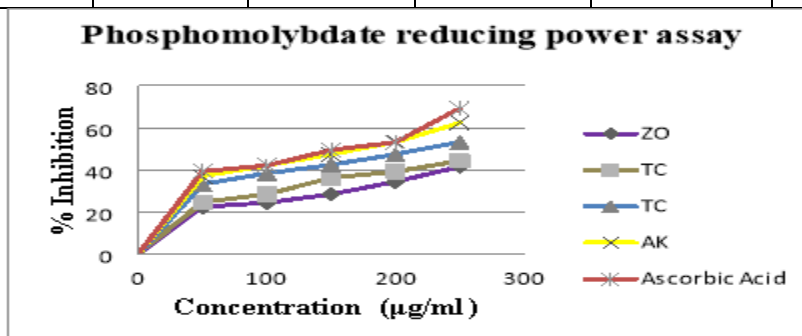
Phosphomolybdate Reducing Power

The phosphomolybdate reducing power of AE of *Zingiber officinale*, *Tinospora cordifolia*, *Terminalia chebula* and AK churna were shown in Figure 9. At the point when the molybdenum (VI) is decreased to Mo (V) by an antioxidant, it shapes a green hued complex at acidic pH within the sight of phosphorous with the retention maxima at 695 nm. The AE of *Zingiber officinale*, *Tinospora cordifolia*, *Terminalia chebula* and AK churna exhibited phosphomolybdate exhibited highest reducing activity 41.44 %, 44.21%, 53.21% and 62.32% at 250 µg/ml whereas for ascorbic acid (standard) it was found to be 69.22 % at 250 µg/ml concentration. The IC_{50} values of AE of *Zingiber officinale*, *Tinospora cordifolia*, *Terminalia chebula* and AK churna was found to be 293.75 (µg/ml), 266.3 (µg/ml), 224.61 (µg/ml) and 174.98 (µg/ml) respectively. Whereas The IC_{50} values of Ascorbic acid (standard) was found to be 158.71 (µg/ml).

However, % inhibition of free radicals in AE of AK churna 62.32 (µg/ml) was lower than reference compound ascorbic acid 69.22 (µg/ml).

Findings of Phosphomolybdate reducing power scavenging activity of Zingiber *officinale*, *Tinospora cordifolia, Terminalia chebula* and *Amrtottara Kwatha* churna are presented in Table 11 and Figure 10.

Table 11: Phosphomolybdate reducing power scavenging activity of *Zingiber officinale*, *Tinospora cordifolia, Terminalia chebula* and *Amrtottara Kwatha* churna

Concentration (µg/ml)	% Inhibition				
	Zingiber officinale	*Tinospora cordifolia*	*Terminalia chebula*	*Amrtottara Kwatha* churna	Ascorbic Acid
0	0	0	0	0	0
50	22.32	24.66	33.19	37.02	39.22
100	24.36	28.33	38.34	42.12	42.03
150	28.32	36.21	42.36	47.36	49.39
200	34.32	39.12	47.37	53.21	53.1
250	41.44	44.21	53.21	62.32	69.22

Fig. 10. Phosphomolybdate reducing activity of *Zingiber officinale* (ZO), *Tinospora cordifolia* (TC)*, Terminalia chebula* (TC) and *Amartottara Kwatha* (AK) churna

Conclusion

In the present work, phytochemical test and quantification of secondary metabolites presented a fact that alkaloid, tannins, saponins and flavonoids are present.The present research work indicates the significant antioxidant potential of AK Churna in all *in vitro* study models. DPPH free radical scavenging model, NO model, H_2O_2 method and phosphomolybdate assay method was employed to screen the free radicals scavenging potential of each ingredient and formulations also. Among all the test ingredients and formulations, AK Churna exhibited highest level of total phenolic contents (488.7 ± 1.07) and the least value for total phenolics was observed for *Zingiber officinale* i.e., 237.64 ± 1.88. This fact concludes that AK Churna is rich in phenolics and may be beneficial for antioxidant action. The present study also confirms the presence of Catechin in AE of AK Churna. The findings suggested the fact that AK churna in the form of kwath may be very beneficial to combat many free radical induced problems. Overall, the study in view of AK churna also suggested that in spite of each ingredient, AK Churna Ayurvedic formulation possess significant antioxidant activity and finally it was concluded that AK churna may be employed in the treatment of several ailments. The proposed mechanism behind the significant antioxidant action may be presence of high level of total phenolic contents.

Acknowledgement:

References

Annur S.; Santosa S.J.; Aprilita N.H. pH dependence of size control in gold nanoparticles synthesized at room temperature. *Oriental J Chemistry*, **2018**, 34(5), 2305.

Baba S.A.; Malik S.A. Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of *Arisaema jacquemontii* Blune, *J Taib Uni Sci*, **2015**, *9*, 449–454.

Badami S.; Channabasavara K.P. *In vitro.* antioxidant activity of thirteen medicinal plants of India's western ghats, *Pharm Bio*, **2007**, *45(5)*, 392–396.

Behera S.K. Phytochemical screening and antioxidant properties of methanolic extract of root of *Asparagus racemosus* Linn, *Int J food proper*, **2018**, *21(1)*, 2681–2688.

Chamundeeswari D.; Kanimozhi P.; Vasanthkumar A.; Reddy C.U. Formulation and evaluation of churna for digestive property, *J Med*, **2007**, 39-43.

Chignola R.; Mainente F.; Zoccatelli G. Rheology of individual chitosan and polyphenol/chitosan microparticles for food engineering. *Foo Hydro*, **2022**, 107869.

Choudhary A.; Sinha M.; Ankalgi A.D.; Goyal K.; Devi A. Analytical Method Development and

Validation for Simultaneous Estimation of Mefloquine Hydrochloride and Artemether in Bulk Drug by Simultaneous Equation Method. *J Applied Pharm Res*, **2022**, *8(4)*, 24-32.

Chaudhary S.K.; Lalvenhimi S.; Biswas S.; Chanda J.; Kar A.; Bhardwaj P.K.; Sharma N.; Mukherjee P.K. High-performance thin-layer chromatography (HPTLC) method development and validation for the quantification of catechin in the hydroalcoholic extract of *Parkia roxburghii* seed. JPC–Journal of Planar Chromatography–Modern TLC, **2022**, 1-7.

Dharmender R.; Madhavi T.; Reena A.; Sheetal A. Simultaneous Quantification of Bergenin, (+)-Catechin, Gallicin and Gallic acid; and quantification of β-Sitosterol using HPTLC from *Bergenia ciliata* (Haw.) Sternb. Forma ligulata Yeo (Pasanbheda). *Pharm Anal Act* **2010**, *1*, 104.

Pratap S. Antioxidant activity of herbal plants: A recent review, *J Drug Dis The* **2013**, *1*, 1-8.

Gharibzahedi S.M.T.; Razavi S.H.; Mousavi S.M. Comparison of antioxidant and free radical scavenging activities of biocolorant synthesized by Dietzia natronolimnaea HS-1 cells grown in batch, fed-batch and continuous cultures. *Indus Crop Prod* **2013**, *49*, 10-16.

Gottumukkala R.V.; Nadimpalli N.; Sukala K.; Subbaraju G.V. Determination of catechin and epicatechin content in chocolates by high-performance liquid chromatography. *Int Sch Res Notice* **2014**, 628196.

Gul R.; Jan Faridullah S.; Sherani S.; Jahan N. Preliminary phytochemical screening, quantitative analysis of alkaloids, and antioxidant activity of crude Plant extracts from *Ephedra intermedia* indigenous to Balochistan. *The Sci World J*, **2017**, 1-7.

Halstead C.W.; Lee S.; Khoo C.S.; Hennell J.R.; Bensoussan, A. Validation of a method for the simultaneous determination of four schisandra lignans in the raw herb and commercial dried aqueous extracts of Schisandra chinensis (Wu Wei Zi) by RP-LC with DAD. *J Pharm Biomed Anal*, **2007**, *45(1)*, 30-37.

Hamidi D.; Aulia H.; Susanti M. High Performance Thin Layer Chromatography: Densitometry Method for Determination of Rubraxanthone in the Stem Bark Extract of *Garcinia cowa* Roxb. *Pharm Res* **2017**, *9(3)*, 230-233.

Hussain M.S.; Fareed S.; Ali M.; Alam S.; Rahman A.; Srivastava A.K. Phytochemical investigation and simultaneous estimation of bioactive lupeol and stigmasterol in Abutilon indicum by validated HPTLC method. *J*

Coast Life Med, **2014,** *2(5)*, 394-401.

Jain D.; Upadhyay R.; Jain S.; Prakash A.; Janmeda P. 2021. TLC and HPTLC Finger Printing Analysis of *Cyperus rotundus* (Linn.). *Lett App NanoBioSci*, **2021,** *11(3)*, 3861-3870.

Jamal M.; Amir M.; Ali Z.; Mujeeb M. A comparative study for the extraction methods and solvent selection for isolation, quantitative estimation and validation of ursolic acid in the leaves of *Lantana camara* by HPTLC method. *Future J Pharm Sci* **2018**, *4(2)*, 229-233.

Khan I.; Sangwan P.L.; Abdullah S.T.; Gupta B.D.; Dhar J.K.; Manickavasagar R.; Koul S. Ten marker compounds-based comparative study of green tea and guava leaf by HPTLC densitometry methods: Antioxidant activity profiling. *J Sep Sci* **2011**, *34(7)*, 749-760.

Khandelwal K.R, Practical Pharmacognosy, Nirali Prakashan, 2008, 19th edition, ISBN: 8185790302.

Krishna K.V.V.S.; Venkatesh B.; Madhavi A.; Devi B.V.; Jaldi C.E.; Bhargavi D.S. Evaluation of Antioxidant Activity of Panchachurnam: An Ayurvedic Polyherbomineral Formulation. *Res J Pharm Tech* **2019**, *12(10)*, 4841-4847.

Kumari N.; Rana R.C.; Sharma Y.P.; Kumar S. Extraction, purification and analysis of sweet compounds in *Stevia rebaudiana* Bertoni using

chromatographic techniques. *Ind J Pharm Sci* **2017**, *79(4)*, 617-624.

Kurutas E.B. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state, Nutri J, **2016**, *15(1)*, 71.

Lourenco S.C.; Martins M.M.; Alves V.D. Antioxidants of natural plant origins: from sources to food industry applications, Molecules, **2019**, *24(22)*, 1-25.

Pal S.K.; Shukla Y. Herbal medicine: current status and the future *Asian Pacific J Cancer Pre* **2003**, *4*, 281-288.

Palma I.G.; Buendia H.B.E.; Alquicira E.P.; Tellez M.T.; Gupta V.K.; Godinez G.D.; Santos J.S. Evaluation of the Antioxidant Activity of Aqueous and Methanol Extracts of *Pleurotus ostreatus* in Different Growth Stages, *Front Micro* **2016**, 7(1099), 1-9.

Patel A.; Patel N.M. Determination of polyphenols and free radical scavenging activity of *Tephrosia purpurea* linn leaves (Leguminosae), *Pharm Res* **2010**, 2(3), 152-158.

Pour B.M.; Jothy S.L.; Latha L.Y.; Chen Y.; Sasidharan S. Antioxidant activity of methanol extracts of different parts of Lantana camara, *Asian Pac J Trop Biomed* **2012**, *2(12)*, 960-965.

Saadati N.; Abdullah M.P.; Zakaria Z.; Sany S.B.T.; Rezayi M.; Hassonizadeh

H. Limit of detection and limit of quantification development procedures for organochlorine pesticides analysis in water and sediment matrices. *Chem Cent J* **2013**, *7(1)*, 1-10.

Shabi R.R.; Vasanthakumari B.; Citarasu T. *In Vitro* Antioxidant activity of various leaf extracts of *Canthium coromandelicum* (Burm F.) Alston, *Asian J Pharm Clin Res,* **2017**, *10(5)*, 214-218.

Sharma V.; Hem K.; Seth A.; Maurya S.K. Standardization and antioxidant activity of an *Ayurvedic* formulation "*Kushavleha*" *Inter J Green Pharm*, **2015**, 9*(4)*, S55-S62.

Tuzimski T. Use of planar chromatography in pesticide residue analysis. Handbook of Pesticides. *Meth Pest Res Anal* **2010**, 187-264.

Venkateswarlu G**.;** Ganapaty S.; Sudhakar A.M. Preparation of *Triphala Churna* using the Ingredients Obtained from Local Market and Comparative Standardization, *Pharm J* **2019**, *11(1)*, 102-111.

Wetchakul P.; Chonsut P.; Punsawad C.; Sanpinit S. LC-QTOF-MS Characterization, AntioxidantActivity, and *In Vitro* Toxicity of Medicinal Plants from the Tri-Than-Thip Remedy, *Evidence-Bas Comp Alter Med*, **2022**, 1-10.