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Ayurvedic polyherbal churna preparation and evaluation for *In-vitro* antioxidant potential

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Abstract
Churna is defined as a fine powder of drug or drugs in Ayurvedic system of medicine. Drugs mentioned in patha, are cleaned properly, dried thoroughly, pulverized and then sieved. Plants *Cucmunis Dipsaceus, Momordica diocia* and *Momordica charantia* var. *muricata* use in formulation is act as immunostimulant, hepatoprotective, antidiabetic, anti-allergic and antiseptic. In Present investigation Polyherbal Churna was prepared and ingredients have been evaluated on the basis of macroscopic evaluation, microscopic evaluation, and determination of p^H, Loss on Drying, Ash Value, Extractive Value, and Powder Flow Property. Thin Layer Chromatography of churna formulation with marker compounds and extracts was studied which confirm the presence of marker compounds in churna and extracts. The antioxidant activity was studied in some of In-vitro antioxidant models like hydrogen peroxide scavenging, ferric reducing power, nitric oxide scavenging activity. On the basis of the results obtained in this present investigation, that formulation show significant antioxidant capacity; it may be due to phytochemicals present in formulation. The results of this study show that the Polyherbal churna can be used as easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry.

Keywords: polyherbal formulation (PHF), antioxidant activity, thin layer chromatography, UV-spectroscopy

Introduction
The traditional medicine is widely used for various human ailments. Traditional system of medicines has become significantly more popular all over the globe because of the effective and curative nature for chronic disease with less toxicity. Indian population even today depends on the Indian system of medicine-Ayurveda ''an ancient science of life'' [1]. Churna is defined as a fine powder of drug or drugs in Ayurvedic system of medicine. Drugs mentioned in patha, are cleaned properly, dried thoroughly, pulverized and then sieved. The churna is free flowing and retains its potency for one year, if preserved in an airtight container. An antioxidant is defined as a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Antioxidant terminates these chain reactions by removing free radical and inhibits other oxidation reactions. Plants contained phenolic, and polyphenol compounds can act as an antioxidant [3].
The plants are selected for research work are *Cucmunis Dipsaceus* belongs to Family-*Cucurbitaceae* locally known as Wild Cucumber contain chemical constituents like alkaloids, flavonoids, tannins, resins, steroids, use as a poultice to treat wound. Juice from the fruit is use as an antidote for poisoning, but has to be supplemented by drinking fresh milk [4]. *Momordica Diocia* belongs to *Cucurbitaceae* locally known as Synonym: kartoli. The green fruit is extensively used as vegetable by cooking or frying. Contain chemical constituents: like alkaloid, flavonoids, glycosides, tannins, saponins and amino acids, triterpene. Use as immunostimulant and antiseptic. Folk remedy for diabetes, antimalerial and antiallergic activity, antioxidant and hepatoprotective activity [5]. *Momordica Charantia Var. Muricata* belongs to Family Family-*Cucurbitaceae* is a tendril bearing medicinally important wine. Medicinal properties of plant includes antimicrobial, antihelminthic, antidiabetic, antifertility [6].

Material and Methods
Chemicals: All chemicals used for the study are purchased from SD-fine chemicals India and all other reagent used were of analytical grade.

Instruments: UV Spectrophotometer (Shimadzu-UV-1601), Centrifuge Machine (Eltek-research centrifuge-TC-4100D).

Methods
Collection and authentication of plants:
The selected plants parts are collected and prepared a herbarium of selected plants and authenticate from botanist material were collected from local areas of Rajuri, District Pune of Maharashtra and identified and authenticated by A Dr. Rahandgule S.S, Professor, and Head of Dept. Of Botany, Balasaheb Jadhav College Of Art, Commerce And Science, Ale Junnar, Pune Maharashtra Dated 20/11/2018. Herbarium specimen has been preserved in laboratory voucher Specimen No. 857 for *Momordica Diocia*. Specimen No. 859 for *Momordica Charantia Var. Muricata* and Specimen No. 858 for *Cucumis Dipsaceus.*

Method of preparation of Polyherbal Churna formulation:
Churna consists of 3 ingredients of Cucurbitaceae family as *Momordica charantia var. muricata* seed, *Momordica diocia* and *Cucumis dipsaceus* fruit powder. All the herbal ingredients of Pharmacopoeial quality mentioned in Table were washed, dried and powdered individually. The powders were completely passed through sieve number 44. Each powdered ingredients were weighed separately, mixed together and pass through sieve number 44 to obtain a homogenous blend [7].

Formula for churna

Table 1: (Formula for churna)

Sr. No.	Ingredient	Quantity
1.	*Momordica Charantia Var. Muricata* powder	30gm
2.	*Momordica Diocia* powder	30gm
3.	*Cucumis Dipsaceus* powder	30gm

Evaluation of churna formulation: The churna formulation was evaluated for different pharmaceutical parameters [8].

Macroscopic evaluation: In macroscopic evaluation the appearance, colour, odour and taste were determined.

Microscopic evaluation: The churna were treated separately with iodine, chloral hydrate, pholorglucinol or potassium iodide with a drop of glycerine. Then observed by binocular microscope attach with camera.

Determination of p^H: The p^H of formulated churna was determined by using digital p^H meter by dissolving 1 gm churna in 100 ml of water.

Loss on drying at 105^0 C: Place about 5 to 10 gm of Powder/drug accurately weighed in a tared evaporating dish. Dry the powder sample at 105^0Cfor 3 hours and weigh. Continue the drying and weighing at half an hour interval until difference between two successive weighing corresponds to not more than 0.25 %.

Determination of Ash Values

1. **Total Ash Value:** 2gm churna was weighed accurately in a previously ignited and tarred silica crucible. The material was then ignited by gradually increasing the heat to 500- 600° C until; it appeared white indicating absence of carbon. Then cooled in a desiccator and total ash of air dried material is calculated.
2. **Acid Insoluble Ash Value:** To the crucible containing total ash, 25ml of HCL was added and boiled gently for 5minutes, and then about 5ml of hot water was added and transferred into crucible. The insoluble matter was collected on an ashless filter paper. This was then washed with hot water until filtrate is neutral and the filter paper along with the insoluble matter was transferred into crucible and ignited to constant weight. The residue was then allowed to cool and then weighed.

Determination of Extractive Values

1. **Water Soluble Extractive Value** 5gms of churna was accurately weighed and placed inside a glass stopper conical flask. It is then macerated with 100ml of chloroform water for 18hours. It was then filtered and about 25ml of filtrate was transferred into a china dish and was evaporated to dryness on a water bath. It was then dried to 105° C for 6hours, cooled and finally weighed.
2. **Alcohol Soluble Extractive Values:** Ethanol was used as solvent in place of chloroform water and remaining procedure was the same as that of water soluble extractive value.

Bulk Density: 10g of churna was taken in a graduated measuring cylinder and tapped on a wooden surface. Bulk density is calculated by using the formula. For the determination of bulk density we use tap density volumetric flask in which we fell the 10g powder sample and calculate the bulk density by using following formula.
Bulk density (g/ml) = weight of sample in gm/ volume occupied by the sample

Tap Density: Tap density of churna was determined after 50 tapping with the help of tap density apparatus. For the determination of tap density we check the tap volume of churna and determine the ratio of weight taken and tap volume of churna sample. The following formula can be used for the determination of tap density.
Tapped density (g/ml) = weight of sample in gm/ volume occupied by the sample

Angle of Repose Angle of repose was determined by using funnel method. The powder was allowed to flow through a funnel fixed on a stand to form a heap. The height and the radius give the angle of repose.

Thin Layer Chromatography

Thin layer chromatography (TLC) studies were carried out for churna, plant extracts, isolated compounds to confirm the presence of different Phytoconstituents in these churna and extracts.
The mobile phase migrates through the stationary phase by capillary action. The separation of solutes takes place due to their differential adsorption / partition coefficient with respect to both mobile and stationary phases. TLC was performed on the marketed pre coated TLC plates (silica gel F-254). The TLC chambers were saturated with the mobile phase and after applying the spot on TLC plates they were kept for development of chromatogram. Then the separations were visualized [9, 10].
The Retention Factor (R_f) was calculated by using following formula:

R_f = Distance travelled by solute from the origin /Distance travelled by solvent from the origin

Table 2: (Thin Layer Chromatography)

Sr. no.	Compound	Test Solution	Stationary Phase	Mobile Phase	Visualization
1.	Cucurbitacin	10mg in 10 ml chloroform	Silica gel G plate	Chloroform: methanol (95:10)	Day light & UV-chamber
2.	Churna formulation				
3.	*Cucumis dipsaceus* extract				
4.	Charantin	10mg in 10 ml Ether	Silica Gel G Plate	Benzene: Methanol (2:8)	Day light & UV-chamber
5.	Churna formulation				
6.	*Momordica charantia var. muricata* extract				
7.	*Momordica diocia* extract				

In-vitro assay for evaluation of antioxidant activity of churna

Scavenging of Hydrogen peroxide

Chemicals: Hydrogen peroxide and Phosphate buffer saline.

Procedure: A solution of hydrogen peroxide (20mM) was prepared in phosphate buffer saline (pH 7.4), different concentrations of plant extract and standard ascorbic acid solution *viz*. 10, 20, 40, 60, 80, 100 µg/ml in methanol (1 ml) where added to hydrogen peroxide solution (2 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used for back ground subtraction.
The percentage inhibition was calculated using the following formula:

% inhibition = [(Abs. control –Abs. sample) /Abs. control] × 100

IC50 represents the level where 50 % of radicals scavenged by test or standard sample [11].

Ferric Reducing Power determination:

Chemicals: Potassium ferricyanide ($K_3Fe(CN)_6$, of purity 98.0%), Ferric Chloride (FeCl3.6H2O, of purity 97.0%), Ascorbic acid (purity 98.3%), Potassium hydrogen phosphate, Sodium hydroxide, Trichloro acetic acid.

Procedure: Different concentrations of plant extract and standard ascorbic acid solution *viz*. 10, 20, 40, 60, 80, 100 µg/ml in 1ml of methanol were mixed with phosphate buffer (2.5ml, 0.2M pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of tricholoroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 g (rpm) for 10 min at room temperature. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride ($FeCl_3$) (0.5 ml, 0.1%) and the absorbance of the reaction mixture indicated increased reducing power. The absorbance was measured at 700nm. All the tests were performed in triplicate and the graph was plotted with the average of three observations [11].
The percentage inhibition was calculated using the following formula:

% inhibition = [(Abs. control –Abs. sample) /Abs. control] × 100

Nitric Oxide Scavenging Assay:

Inhibition of nitric oxide radical production was estimated using the Griess reaction method. Griess reagent was prepared by mixing an equal volume of 1% sulphanilamide in 5% v/v phosphoric acid and 0.01% naphthylethylenediamine (NEDA) in distilled water was added. The solution of sodium nitroprusside (5mM) in standard phosphate buffer (0.025 M, pH 7.4) was prepared. Different concentrations of standard or test sample: 10, 20, 40, 80 and 100 µg/ml was incubated with sodium nitroprusside solution at 37°C for 5 h. 0.05 ml of each sample was mixed with 0.5 ml of Griess reagent. The absorbance of chromophore formed during the digitization of nitrite with sulphanilamide and its subsequent coupling with NEDA was read at 546 nm. BHT was used as a standard [12].
The percentage inhibition of nitric oxide as stable nitrite/nitrate product was calculated using the following formula:

% NO inhibition = [(Abs. control –Abs. sample) /Abs. control] × 100

Total Antioxidant capacity

For total antioxidant capacity assay, 0.3 ml of the churna extract (10 mg/ml) dissolved in water and mixed with 3 ml of reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) in Eppendorf tube. The tubes were capped and incubated in a thermal block at 95 °C for 90 min. After 90 min, the mixture was cooled to room temperature; the absorbance was measured at 695 nm against reagent blank. Methanol (0.3 ml) in the place of extract is used as the blank. BHT was used as the standard and the total antioxidant capacity is expressed as equivalents of BHT [11].

Result and Discussion

Preparation of Churna Formulation: Polyherbal churna formulation is prepared as per given in formula.

Fig 1: (formulation)

Evaluation of churna formulation: The churna formulation was evaluated for different pharmaceutical parameters.

Macroscopic evaluation:

Result: The Churna formulation were macroscopically evaluated and the obtain result as shown in table no.3

Table 3: (Macroscopic evaluation)

Sr.No.	Parameter	Obtain Result
1.	Appearance	Smooth /fine
2.	Colour	Yellowish –brown
3.	Odour	Unpleasant
4.	Taste	Bitter

Microscopic evaluation

Result: Microscopically the churna contain xylem vessel, lignified sclerides, Endosperm, strach grain and epidermis as shown in fig. no.2

Powder microscopy of churna formulation:

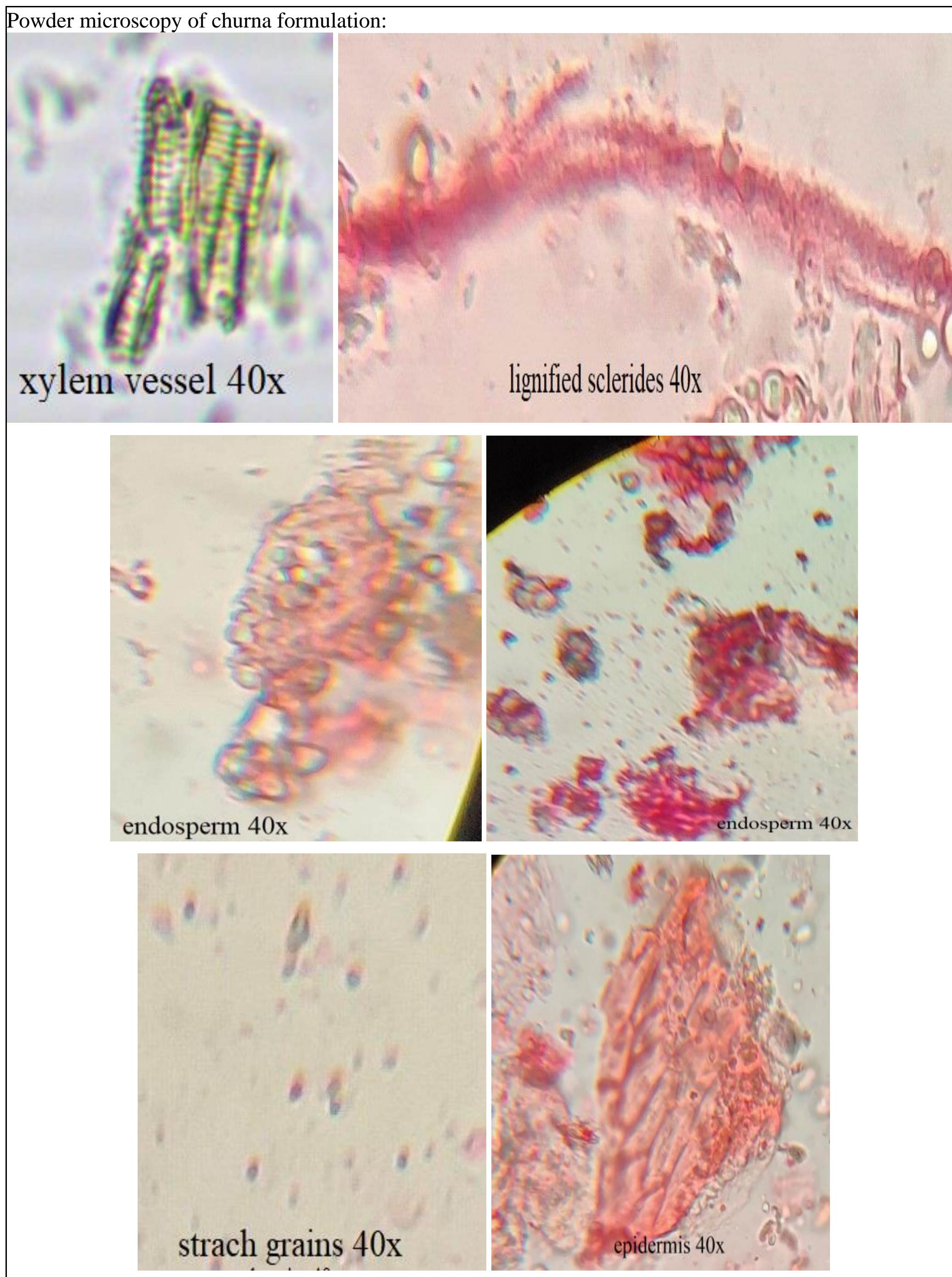


Fig 2: (Microscopic evaluation)

Determination of pH

Result: The Churna were evaluated for pH and result found to be 6.80.

Loss on drying at 105⁰ C

Result: The Churna formulation were evaluated for Moisture Content and the result found to be 11.5%w/w.

Determination of Ash Values

Result: The Churna formulation were evaluated for Ash Value and result shown in Table No.4

Table 4: (Determination of Ash Values)

Sr. no.	Parameter	Obtain value
1.	Total Ash Value	8.275%w/w
2.	Water Soluble Ash value	5.26% w/w
3.	Acid Insoluble Ash Value	0.84%w/w

Determination of Extractive Values

Result: The Churna formulation were evaluated for extractive value determination and the obtain result as shown in table. No.5

Table 5: (Determination of Extractive Values)

Sr. No.	Parameter	Obtain Value
1.	Water soluble extractive Value	5.02%w/w
2.	Alcohol soluble extractive Value	1.04%w/w

Flow Property

Result: Flow property of churna were evaluated by Bulk Density, Tap Density and Angle of Repose determination and the obtain result as shown in table. No.6

Table 6: (Flow Property)

Sr. no.	Parameter	Obtain value (gm/ml)
1.	Bulk Density	0.36
2.	Tap Density	0.67
3.	Angle of Repose	40°

Thin Layer Chromatography

Result: The Churna formulation were evaluated for thin layer chromatography determination and the obtain result as shown in Figure. No.3, 4 And Table No.7, 8

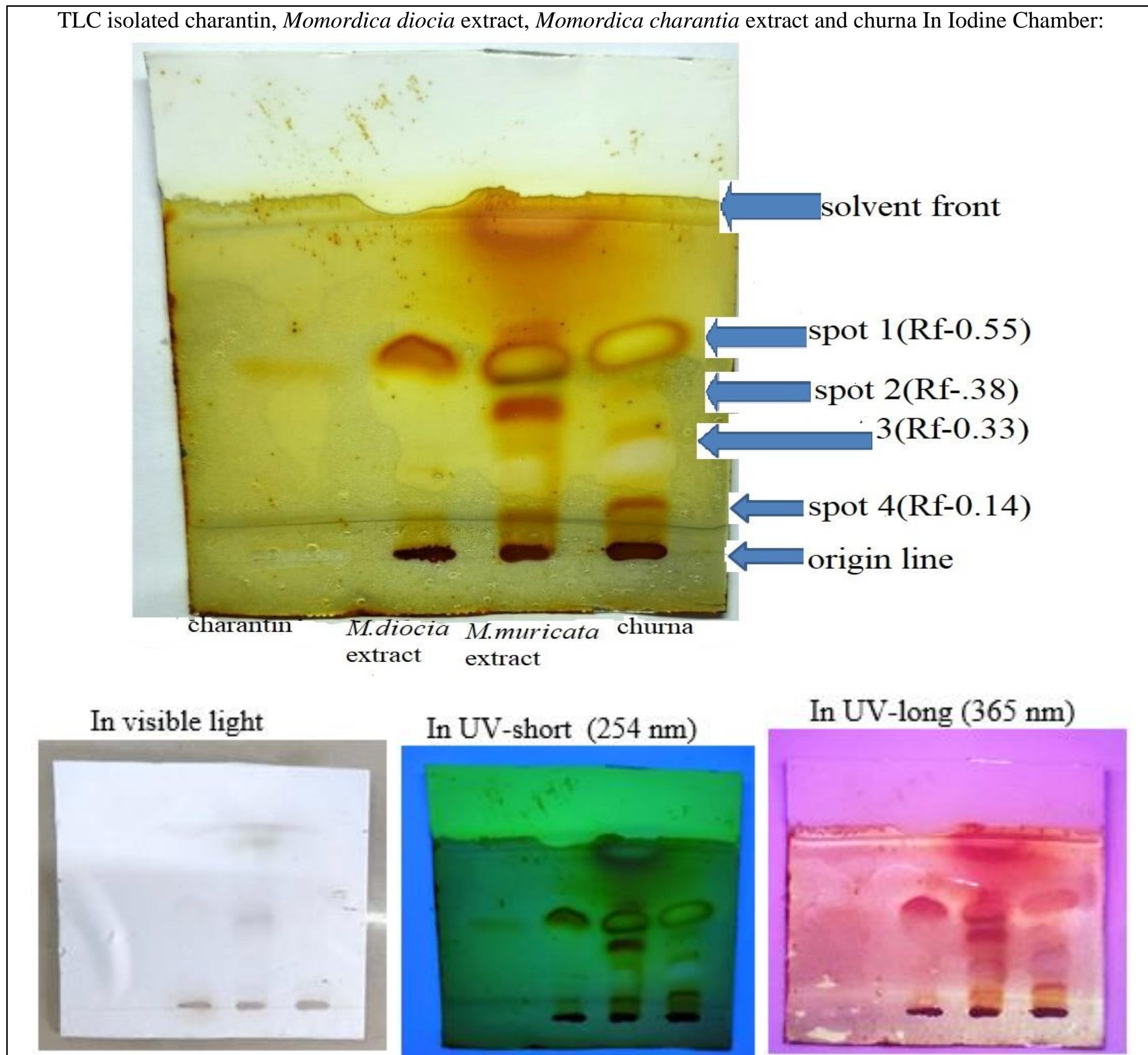


Fig 3: (Thin Layer Chromatography)

Table 7: (Thin Layer Chromatography)

Sr. no.	Name of compound	No. of spot	Rf-value	
			Obtain	Standard(Ref.no.10)
1.	Isolated charantin	1	0.53	05
2.	*Momordica diocia* extract	1	0.54	0.5
3.	*Momordica char. var.muricata* ext.	3	0.53	0.5
4.	Churna formulation	1	0.55	0.5
		2	0.38	
		3	0.33	
		4	0.14	

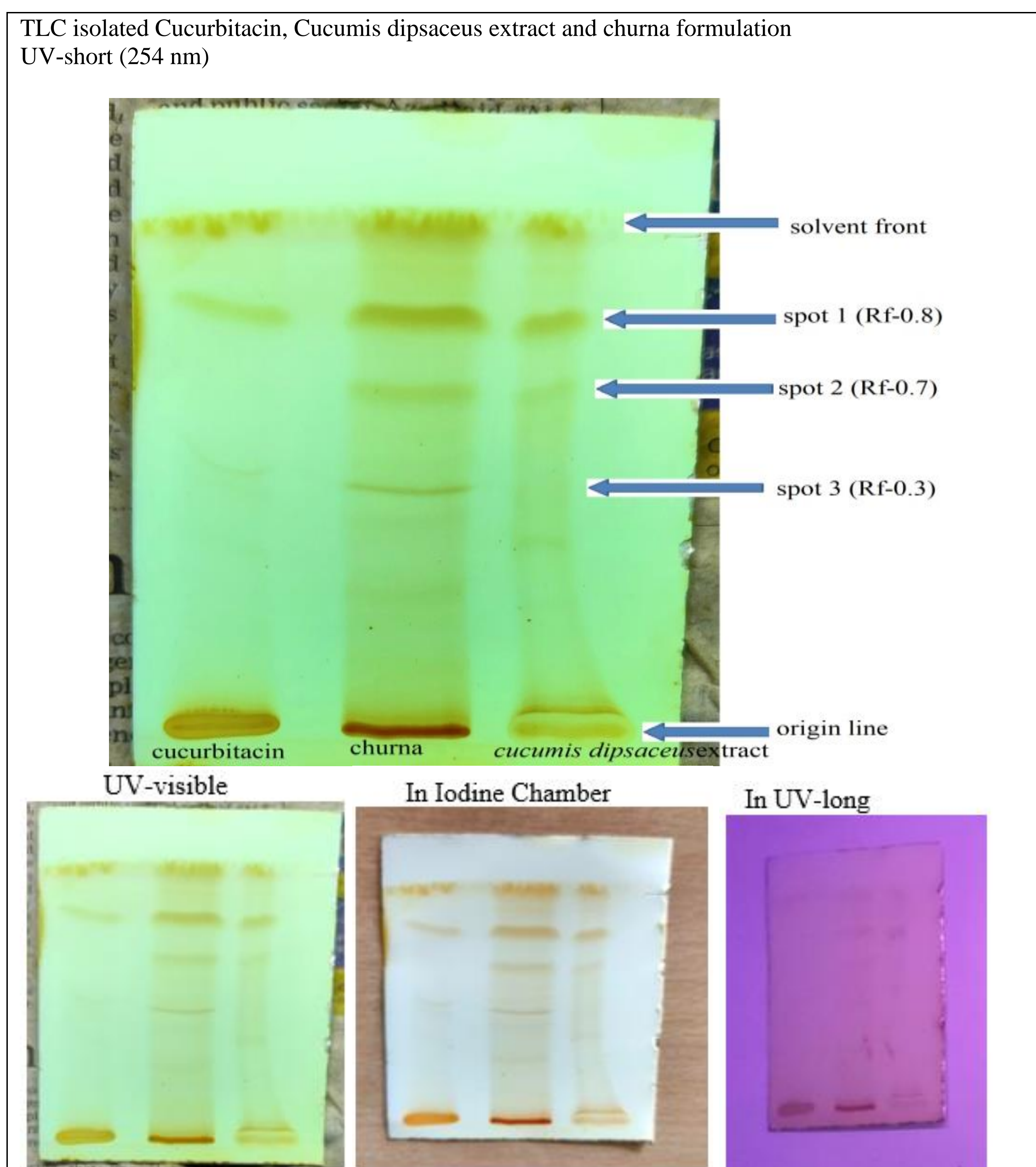


Fig 4: (Thin Layer Chromatography)

Table 8: (Thin Layer Chromatography)

Sr. no.	Name of compound	No. of spot	Rf-value	
			Obtain	Standard (as per Ayurvedic pharmacopeia of India)
1.	Isolated cucurbitacin	1	0.83	0.9
2.	Churna formulation	1	0.83	0.9
		2	0.7	
		3	0.5	
3.	*Cucumis dipsaceus* extract	1	0.83	0.9
		2	0.7	

***In-Vitro* Assay for Evaluation of Antioxidant Activity of Churna**

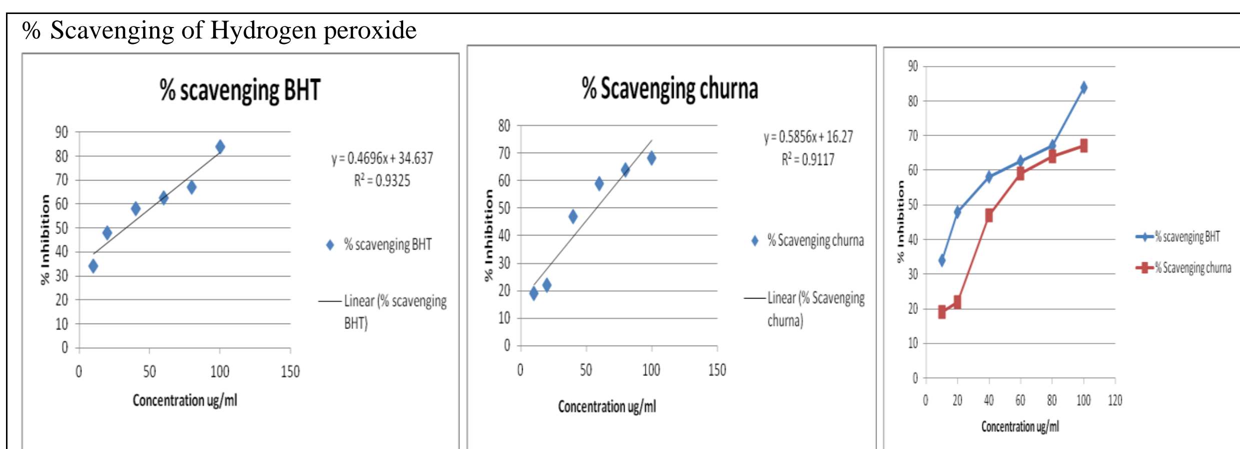
Result

Hydrogen Peroxide: The maximum hydrogen peroxide scavenging activity of churna 68.15 % was observed at 100 µg/ml concentration and the IC_{50} value of churna and std. BHT butyrated hydroxy toluene were found to be 58.03µg/ml and 32.75µg/ml respectively as shown in table no.9, 10 and graph no.1.

Table 9: (Absorbance at different concentration and scavenging %)

Conc. (µg/ml)	Absorbance at 230 nm		Scavenging (%)	
	Churna	BHT	Churna	BHT

10	2.284	1.861	19	34
20	2.191	1.456	22	48
40	1.495	1.179	47	58
60	1.145	1.062	59	62.59
80	1.014	0.931	64	67.20
100	0.904	0.460	68.15	83.79



Graph 1: (%Scavenging of Hydrogen peroxide)

Table 10: (Regression equation, Regression coefficient and IC_{50} value of radical scavenging models)

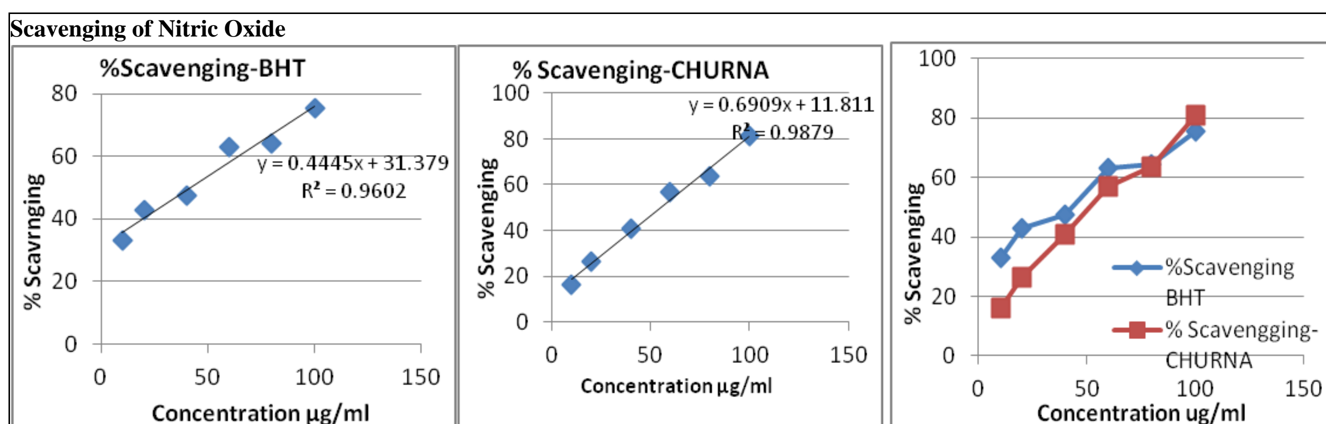
Data	Hydrogen Peroxide scavenging		Nitric Oxide Scavenging		Ferric Reducing Assay	
	CHURNA	BHT	CHURNA	BHT	CHURNA	BHT
Regression equation	$y = 0.5856x + 16.27$	$y = 0.4696x + 34.637$	$y = 0.6909x + 11.811$	$y = 0.4445x + 31.379$	$y= 0.7448x + 17.321$	$y= 0.9211x + 24.653$
Regression coefficient	$R^2 = 0.9117$	$R^2 = 0.9325$	$R^2 = 0.9879$	$R^2 = 0.9602$	$R^2 = 0.9588$	$R^2 = 0.9805$
IC_{50} Value (µg/ml)	58.03µg/ml	32.75µg/ml	55.274 µg/ml	42.34 µg/ml	43.899 µg/ml	27.52 µg/ml

Nitric Oxide Scavenging Assay:

Result: The maximum Nitric Oxide scavenging activity of churna 81.04% was observed at 100 µg/ml concentration and the IC_{50} value of churna and std. BHT butyrated hydroxyl toluene were found to be 55.274 µg/ml and 42.34 µg/ml respectively as shown in table no.10,12 and graph no.3

Table 11: (Absorbance at different concentration and scavenging %)

Conc. (µg/ml)	Absorbance at 546 nm		Scavenging (%)	
	Churna	BHT	Churna	BHT
10	0.915	0.731	16.20	33.05
20	0.802	0.623	26.55	42.94
40	0.648	0.575	40.65	47.34
60	0.469	0.401	57.05	63
80	0.398	0.390	63.55	64.28
100	0.207	0.268	81.04	75.45

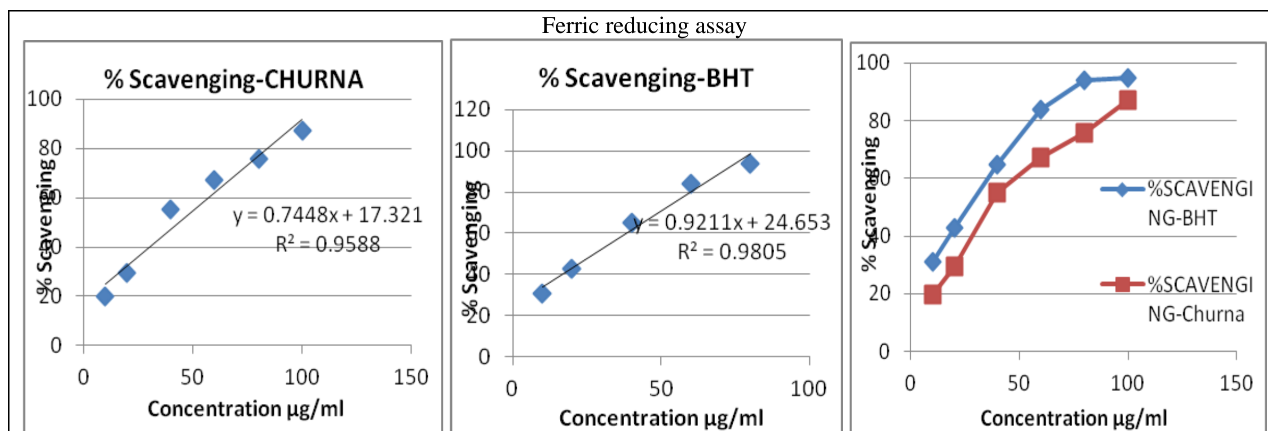


Graph 2: (% Scavenging of nitric oxide)

Ferric reducing assay: the maximum Ferric reducing activity of churna 87.30% was observed at 100 µg/ml concentration and the IC_{50} value of churna and std. BHT (butyrated hydroxyl toluene) were found to be 43.899 µg/ml and 27.52 µg/ml respectively as shown in table no.10,14 and graph no.3

Table 12: (Absorbance at different concentration and scavenging %)

Conc. (µg/ml)	Absorbance at 700 nm		Scavenging (%)	
	Churna	BHT	Churna	BHT
10	1.553	1.338	19.86	30.95
20	1.369	1.107	29.36	42.87
40	0.870	0.681	55.10	64.86
60	0.634	0.310	67.28	84
80	0.467	0.116	75.90	94.01
100	0.246	0.092	87.30	95



Graph 3: (% Scavenging of ferric reducing assay)

Total Antioxidant capacity:

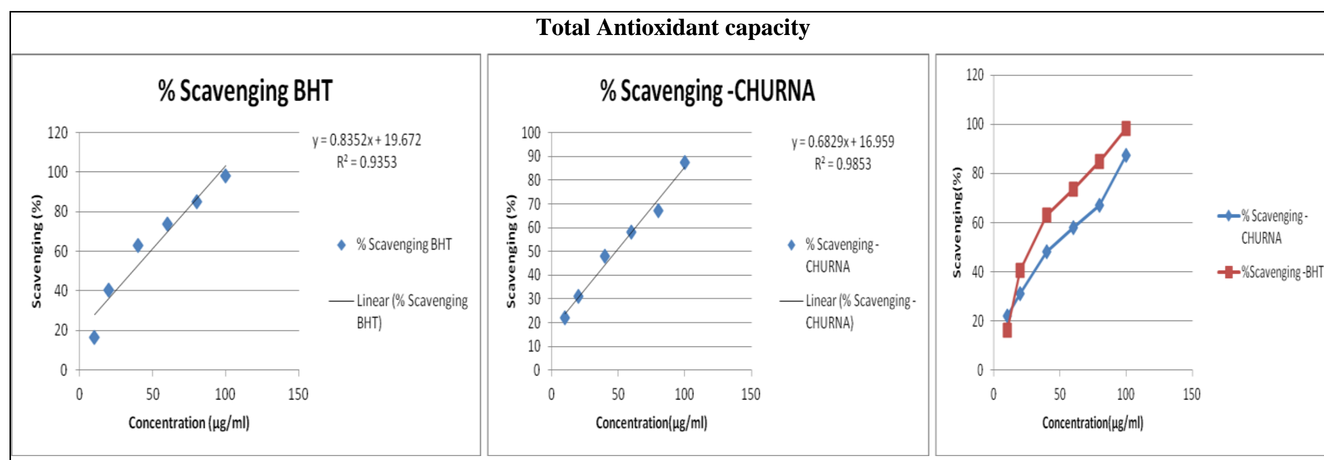
Result: The total antioxidant capacity in the churna formulation was determined by the method based on the formation of the phosphomolybdenum complex measured spectrophotometrically at 695 nm[1] IC_{50} value of churna is 48.38µg/ml and IC_{50} value of BHT is 36.31µg/ml.

Table 13

Conc. (µg/ml)	Absorbance at 695 nm		Scavenging (%)	
	Churna	BHT	Churna	BHT
10	1.217	1.304	21.93	16.35
20	1.071	0.929	31	40.41
40	0.803	0.090	48	63.11
60	0.648	0.417	58	73.67
80	0.513	0.227	67.09	85
100	0.196	0.025	87.42	98.39

Table 14: (Regression equation, Regression coefficient and IC_{50} value of radical scavenging models)

TAC	BHT	Churna
Regression equation	y = 0.8352x + 19.672	y = 0.6829x + 16.959
Regression coefficient	R^2 = 0.9353	R^2 = 0.9853
IC_{50} Value (µg/ml)	36.31µg/ml	48.38µg/ml



Graph 4: (% Scavenging of total antioxidant capacity)

Conclusion

Ployherbal churna was prepared and evaluated with macroscopical, microscopical and powder flow property. TLC of churna formulation and extracts confirms the quality and purity of plant and its identification. In antioxidant activity by inhibiting hydrogen peroxide scavenging the maximum scavenging of churna 68.15 % was observed at 100 µg/ml concentration and the IC_{50} value of churna and std. BHT butyrated hydroxy toluene were found to be 58.03µg/ml and 32.75µg/ml respectively. In ferric reducing power the maximum Ferric reducing activity of churna 87.30% was observed at 100 µg/ml concentration and the IC_{50} value of churna and std. BHT (butyrated hydroxyl toluene) were found to be 43.899 µg/ml and 27.52 µg/ml respectively. In nitric oxide scavenging activity maximum Nitric Oxide scavenging activity of churna 81.04% was observed at 100 µg/ml concentration and the IC_{50} value of churna and std. BHT butyrated hydroxyl toluene were found to be 55.274 µg/ml and 42.34 µg/ml respectively.

Total antioxidant capacity was also measured Spectrophotometrically at 695 nm[1] which is found to be IC_{50} value of churna IS 48.38µg/ml and IC_{50} value of BHT is 36.31µg/ml. which may due to phenolic triterpenoids like cucurbitacins and steroidal glycosides like charantin which are potent compounds. Further studies needed to evaluate the *in vivo*-antioxidant potential of churna formulation in various animal models.

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