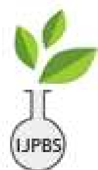

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ESTABLISHING CORRELATION OF THERAPEUTIC ACTIVITY OF A SIDDHA FORMULATION WITH ITS ANTIOXIDANT ACTIVITY. A COMPARATIVE STUDY.

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ABSTRACT

Scientific documentation of traditional system of medicine is increasing and need for preparing it for Siddha formulation has become the need of the hour. Amukkara choornam is a popular poly herbal Siddha formulation composed of spices and herbs. It is a most effective formulation used during Gastric troubles, spleen enlargement, leucorrhoea, hiccup, anemia, tuberculosis and kappa diseases. Amukkara choornam samples were collected from different manufacturers of Tamilnadu, India labeled as A, B, C and in house preparation as D. The methanolic extracts of samples were evaluated for their total phenolic content and invitro antioxidant activity by Electrochemical measurement, Total antioxidant capacity (Phosphomolybdenum reduction Assay), Iron (III) to Iron (II) reduction assay, 1, 1'-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method and Reducing power. In all cases sample-A, B, C and D shows good antioxidant activity but Sample-C shows more activity as compared to other samples.

KEY WORDS

Antioxidant activity, Amukkara choornam, DPPH radical, oxidation potential, Reducing power, BHT equivalent, relative reductive efficiency.

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen have been implicated in degenerative disease such as cancer, inflammation, atherosclerosis and ageing [1, 2]. Antioxidants are compounds that inhibit or delay oxidation of other molecules by terminating initiation or propagation of oxidizing chain reactions [3].A great number of spices and aromatic herbs contain chemical compounds exhibiting antioxidant properties [4]. These properties are attributed to a variety of active phytochemicals including vitamins, carotenoids, terpenoids,alkaloids, flavonoids, lignans, simple phenols and phenolic acids, etc [5]. Amukkara choornam is a popular poly herbal Siddha formulation composed of spices and herbs like *Syzygium aromaticum, Cinnamomum wightii, Elettaria cardamomum, Piper nigrum, Piper longum, Zingiber officinale, Withania somnifera* and Cane sugar in geometric progression. It is a most effective formulation used during gastric troubles, spleen

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enlargement, leucorrhoea, hiccup, anemia, tuberculosis and kappa diseases [6].

Herbal drug technology is used for converting botanical materials into medicines, where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge is important. So to establish correlation between the above therapeutic activity and antioxidant activity, In the present paper we evaluated the invitro antioxidant capacities of methanolic extract of various samples of Amukkara choornam collected from different manufacture of Tamilnadu, India and Labeled as A, B, C and in-house preparation as D. (Table-1) Five different assays (Measurement of oxidation potential by cyclic voltametry, reduction of transition metal ions by Phosphomolybdenum complex, ferric to ferrous reductive activity, 1, 1'-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity and Reducing power) were used. Antioxidant activities of the various methanolic extract of samples were compared with those of standards i.e. gallic acid and BHT.

MATERIALS AND METHODS

Materials

The samples were collected from the manufacturers of different regions in South India (Table-1). For in house preparation samples of raw materials were collected from local raw traders of Ranchi, Jharkhand, India. The species of samples were identified by Dr S. Jha (Department of pharmaceutical sciences, Birla institute of Technology, Mesra, Ranchi, India), according to morphological characteristics. The formulation was prepared as per Siddha formulary.

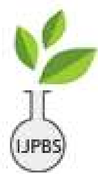
Table 1.

Samples of Amukkara Choornam

Sample	Origin
A	Aravindh Herbal laboratories (P) Ltd
B	Rajapalayam (south India)
C	SKM Siddha and Ayurvedic Medicines India (P) Ltd Erode.
D	In House preparation

Chemicals and reagents

Potassium ferricyanide, ferrous chloride, ferric chloride, Folin–Ciocalteu's reagent (FCR), methanol, sulphuric acid, Potassium chloride, Sodium phosphate, ammonium molybdate and trichloroacetic acid (TCA) were purchased from E. Merck (Darmstadt, Germany). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Butylated Hydroxyl Toluene (BHT), Ascorbic acid (AA) and gallic acid were purchased



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from Sigma Chemical Co. (Sigma–Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents are of analytical grade.

Preparation of extract

About 10 gm of powder was taken in a beaker extracted with 30ml of methanol with the help of sonicator for 30 minutes. The solvent recovered was concentrated to yield a residue which was stored in the desiccators for use in subsequent experiments.

Total Polyphenol estimation

The amount of total polyphenlos in the methanolic extract of Amukkara choornam was estimated according to Folin-Ciocalteu method [7]. Different concentrations of Gallic acid (50µg/ml-250 µg/ml) were prepared for standard curve. 0.1 ml of these different concentrations of Gallic acid was taken in different test-tubes and to it 0.5 ml undiluted Folin-Ciocalteu reagent was added. After 1 min, 1.5 ml 20 % (w/v) anhydrous sodium carbonate (Na_2Co_3) were added and volume was made up to 10 ml with water. After 1 hour incubation at 25 degree centigrade, the absorbance was measured at 760nm. Plot of absorbance versus concentration was plotted to prepare the standard curve. 100 mg of methanolic extract of various formulations and in home preparation was taken to which 100 ml of methanol was added; 0.1 ml of extract was taken and followed as above. The results are means of three repetitions expressed in the form of gallic acid equivalents per g of extract.

Cyclic voltametry

A Cyclic voltammeter (Ecochemie, Twente, model PGSTAT 30, Holland) with three electrode system viz. Calomel as reference electrode, a Platinum electrode as working electrode and a platinum wire as a counter electrode was used for our experiment. Cyclic voltametry tracing were recorded from 0.2 to 1 V at a scan rate of 100mV/s [8]. Data were analyzed using GPES 3.2 software from Ecochemie, running on a P-III personal computer.

Total antioxidant capacity (Phosphomolybdenum reduction Assay)

This method of Prieto, Pineda and Aguilar (1999) was used [9]. Total antioxidant capacity was measured by spectrophotometric method. 0.1ml of the extract (10mg/ml) dissolved in water was combined in eppendorf tube with 1 ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95^0C for 90 min. After cooling to room temperature; the absorbance of the aqueous solution of each was measured at 695nm against blank. Total antioxidant capacity in terms of BHT equivalent were determined.

Iron (III) to Iron (II) reduction assay

The reductive capacities of the methanolic extracts were assessed using ferric to ferrous reductive activity as determined spectrophotometrically from the formation of perl,s Prussian blue colored complex [10]. 1 ml of each extract in water was mixed with 2.5ml phosphate buffer (0.2M, PH 7.0) and 2.5ml of a 1% (w/v) potassium hexacyanoferrate [K_3Fe $(CN)_6$] solution. After 30 min incubation at 50^0c, 2.5ml (10%w/v) tri-chloroacetic acid was added and the mixture was centrifuged for 10 min (1800 rpm). Finally, 2.5ml of the upper layer were mixed with 2.5ml water and 0.5ml (0.1%, w/v) ferric chloride and the absorbance was recorded at 700nm. BHT was

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taken as standard. Finally relative reductive efficiency (RRE) was calculated by taking the ratio of the slope of sample to that of BHT.

DPPH radical scavenging effect

The DPPH assay was carried out by Brand-Williams and his co-workers (1995) [11]. 25, 50, 75, 100, 250, 500 µg of the sample and standard solution was prepared in methanol. 4 ml of various concentrations of the extracts in methanol was added to a 1 ml solution of DPPH radical in methanol (final concentration of DPPH was 0.2 mM). The mixture was shaken vigorously and allowed to stand for 30 min in room temperature the absorbance of the resulting solution was measured at 517 nm with a spectrophotometer (Shimadzu UV-1601, Kyoto, Japan). Inhibition of free radical DPPH in percent (I %) was calculated in following way

$$I(\%) = 100 \times (A_0 - A_1) / A_0$$

Where A_0 is the absorbance of the control reaction (containing all reagents except the test compound), and A_1 is the absorbance of the test compound. BHT and Ascorbic acid was used as a control.

Reducing power

The reducing power was determined according to the method of Oyaizu (1986). Each extract (0.5mg/ml–20mg/ml) in methanol (1 ml) was mixed with 2.5 ml of 200 m M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide and the mixture was incubated at 50^{0}C for 20 min. Then, 2.5 ml of 10% trichloroacetic acid was added, the mixture was centrifuged at 200 g (MSE Mistral 2000, London, UK) for 10 min [12]. The upper layer (2.5 ml) was mixed with 2.5 ml of de-ionized water and 0.5 ml of 0.1% ferric chloride and the absorbance was measured at 700 nm in a UV-visible spectrophotometer.BHT was used as standard.

RESULT AND DISCUSSION

Total phenolic content in methanolic extract of different samples of Amukkara choornam as estimated by Folin-ciocalteu Reagent method in the present study shows that different samples show difference in gallic acid equivalent (Table-2). This is due to vary in nature of active ingredients in various samples.

Table-2

Gallic acid equivalent of different samples of Amukkara choornam.

Sl.no.	Sample name	Gallic acid equivalent(Mean±SEM)
1	Sample-A	31.79±0.308
2	Sample-B	19.65±0.284
3	Sample-C	61.78±0.880
4	Sample-D	25.03±0.290

The redox properties are crucial for better understanding of the electron transfer process. Cyclic voltammetry is an established instrumental tool for the measurement of electron transfer efficiency and in turn antioxidant efficacy of a test compound. Thus, we have measured the oxidation potential of the different extract of Samples as well as the individual ingredients. By and large lower the oxidation potential of a test compound higher the antioxidant efficacy. The low oxidation potential value of the methanolic

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extract confirmed that these formulations have efficient oxidative radical scavenger. (Fig. 1) Sample-C shows good anti oxidant activity than other samples.

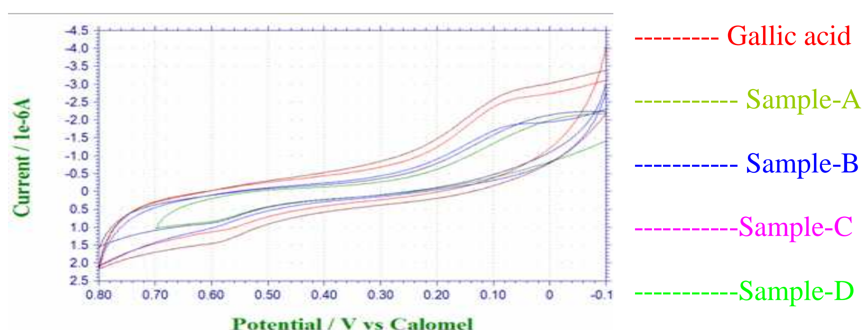

Fig.1

Cyclic Voltammogram of methanolic extract of Amukkara choornam.

The different Amukkara choornam samples were also used to determine their antioxidant capacities by the formation of green phosphomolybdenum complex. The formation of the complex was measured by the intensity of absorbance in extracts at the concentration of 5mg/ml. The results indicate that under these conditions methanolic extracts of Amukkara choornam are powerful antioxidants (Table-3). However, the differences in the degree of molybdenum reduction were observed. This is due to vary in content of total phenolics as established in total phenolic compound estimation.

Table 3.

Total anti-oxidant activity in terms of Equivalents of BHT

Sl.no.	Sample name	BHT equivalent (mg/mg)
1	Sample-A	0.386± 0.0005
2	Sample-B	0.365±0.0008
3	Sample-C	0.4025±0.0024
4	Sample-D	0.377±0.0005

In the ferric to ferrous reduction assay the electron donation capacity (reflecting the reductive power) of the sample were assessed and compared to that of BHT, which is known to be a strong reducing agent. All the extract showed some degree of electron donation capacity in terms of RRE (Table-4) but Sample-C shows good activity than others due to higher content of phenolic compounds.

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Table-4

RRE of various samples with respect to BHT.

Sample	RRE	Slope	Intersept ($\times 10^{-3}$)	R^2
BHT	1	0.0599	6.3	0.9981
Sample-A	0.5409	0.0324	1.8	0.9994
Sample-B	0.4073	0.0244	1.9	0.9969
Sample-C	0.7362	0.0441	8.0	0.9930
Sample-D	0.4257	0.0255	1.7	0.9988

Figure 2. shows the DPPH radical scavenging activity of different Amukkara choornam samples with various concentrations. As positive control, BHT and Ascorbic acid were also examined. Sample-C showed the best result through all concentration of DPPH assay. At lower concentration less difference in DPPH scavenging activities was observed between standard and samples. How ever, as concentration increases the difference in scavenging activities between BHT, Ascorbic acid and different samples become more significant.

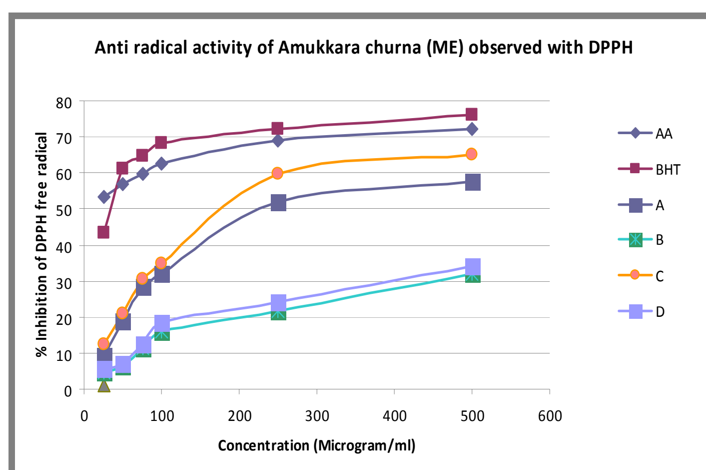

Fig 2. ***Anti radical activity of Amukkara choornam observed with DPPH.***

Reducing powers in terms of EC_{50} value of various samples of Amukkara choornam were excellent and were in the range of 12.65-13.85, comparable with that of BHT (12.26) (Table-5).The reducing powers of the samples due to the hydrogen donating abilities.

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Table 5.

EC50 Value of Different samples With respect to BHT.

Sl.no.	Sample name	EC 50(mg/ml)±SEM
1	Sample-A	13.85±0.3774
2	Sample-B	17.37± 0.4573
3	Sample-C	12.65± 0.6599
4	Sample-D	13.36±0.5861
5	BHT	12.26±0.6630

Ayurvedic formulations claimed to be made according to CCRAS guidelines and effective but it is very difficult to maintain uniformity in formulations which is may be due to the natural heterogeneity, the quality of herbal starting material obtained from wild collections shows more and more fluctuations. [13] The different anti-oxidant activity of different formulations may be due to vary in the content of polyphenolic constituents. Which can be further studied for quantitative estimation of active constituents by analytical methods and also its pharmacological activity by different models.

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