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INVITRO ANTIOXIDANT PROPERTIES OF SIDDHA SASHTHIRIA MEDICINE SANGU CHUNNAM

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ABSTRACT

Chunnam are exclusive Siddha metallic/minerals, Animal's derivative such as horns, shells, feathers grounding and preserved with herbal juice or decoction and exposed for siddha, which are widely recommended for treatment of a variety of chronic ailments. Sangu Chunnam is an animal product *Sangu (Bivalvae Shells)*, which prepared by the methods recommended in the Siddha literature using *lemon Juice (Citrus lemon)*. The physical properties of Sangu Chunnam solubility, colour and pH and Infrared (IR) spectroscopic were analysed. The IR spectrum the functional groups were acknowledged and they are correlated with the curative nature of the drug. Invitro antioxidant activities of Sangu Chunnam were investigated using ABTS assays, lipid peroxidation, metal chelating, nitric oxide scavenging and superoxide scavenging assay. The results confirmed that the Sangu Chunnam synthesized by lemon juice were crystalline in nature. ABTS radical concentration (EC_{50} 15.24 µg/mL), Lipid peroxidation concentration (EC_{50} 18.23 µg/mL), Metal chelating concentration (EC_{50} 16.4 µg/mL), nitric oxide radical concentration (EC_{50}16.63 µg /mL) and superoxide scavenging activity (EC_{50} 19.36 µg/mL) under *in vitro* conditions. The antioxidant methods confirmed that the Sangu Chunnam have more antioxidant activity as compared to vitamin C. The studied Sangu Chunnam possess considerable antioxidant activities and may contribute to the wellbeing of individuals who possess aging related diseases.

KEYWORDS: Anti Oxidant, Five Methods, Sangu Chunnam, Siddha Preparation, Vitamin-C Enriched.

INTRODUCTION

In Siddha traditional medicine plays an integral role in providing basic health care needs of the population. WHO defines traditional medicine as the broad set of health care practices developed over a long period and may be used in conjunction with allopathic medicines for maintenance of health as well as in the prevention, improvement, diagnosis or treatment of physical and/or mental illness (Ravishankar and Shukla, 2007). On the inside, free radicals are formed as a normal part of breakdown within the mitochondria, through peroxisomes, xanthine oxidase, inflammation processes phagocytosis, arachidonate pathways, ischemia, and physical exercise. Peripheral factors that comfort to promote the production of free radicals are smoking, environmental pollutants, radiation, drugs, pesticides residue, industrial solvents and ozone. It is ironic that these elements, essential to life (especially oxygen) have harmful effects on the human body through these reactive species (Lobo et al., 2010). The stability concerning the production and neutralization of ROS by antioxidants is very gentle, and if this equilibrium inclines to the overproduction of ROS, the cells start to suffer the significances of oxidative stress (Wiernsperger, 2003). It is estimated that every day a human cell is targeted by the hydroxyl radical and other such species and average of 105 times inducing oxidative stress (Valko et al., 2004). The main targets of ROS, RNS and RSS are proteins, DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) molecules, sugars and lipids (Craft et al., 2012).

The damage induced by free radicals to DNA can be described both chemically and structurally having a characteristic pattern of modifications: Production of base-free sites, deletions, modification of all bases, frame shifts, strand breaks, DNA–protein cross-links and chromosomal arrangements. An important reaction involved with DNA damage is the production of the hydroxyl radical through the Fenton reaction. This radical is known to react with all the components of the DNA molecule: the purine and pyrimidine bases as well as the deoxyribose backbone. The peroxyl and OH-radicals also intervene DNA oxidation (Valko et al., 2004). Regarding sugars, the formation of oxygen free radicals during early glycation could contribute to glycoxidative damage. During the initial stages of non-enzymatic glycosylation, sugar fragmentation produces short chain species like glycolaldehyde whose chain is too short to cyclize and is therefore prone to autoxidation, forming the superoxide

radical. The resulting chain reaction propagated by this radical can form a and b-dicarbonyls, which are well known mutagens (Benov and Beema, 2003).

MATERIALS AND METHODS

ABTS (2,2'-AZINO-BIS-3-ETHYL BENZTHIAZOLINE-6-SULPHONIC ACID) RADICAL SCAVENGING ASSAY

ABTS radical scavenging activity of Sangu chunam was followed by Re et al. (1999). ABTS radical was newly prepared by addition 5 ml of 4.9 mM potassium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with distilled water to produce an absorbance of 0.70 at 734 nm and the same was used for the antioxidant activity. The final solution of standard group was made up to 1 ml with 950 µl of ABTS solution and 50 µl of Ascorbic acid. Correspondingly, in the experiment group, 1 ml reaction mixture encompassed 950 µl of ABTS solution and 50 µl of different concentration of each extracts. The reaction mixture was vortexed for 10 s and after 6 min, absorbance was recorded at 734 nm against distilled water by using a Deep Vision (1371) UV–Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound.

ABTS Scavenging Effect (%) = $[(A_0 - A_1/A_0) \times 100]$ Where A_0 is the absorbance of the control reaction and A_1 is the absorbance of extract.

INHIBITION OF LIPID PEROXIDATION ACTIVITY

Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa et al. (1979). The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH_4)_2SO_4.7H_2O$ (0.06 mM); and different concentrations of sangu chunam in a final volume of 0.5 ml. The experimental mixture was incubated at 37°C for 1 h. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify

TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard.

Inhibition of lipid peroxidation (%) by the each extracts was calculated according to 1-(E/C) × 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample.

SUPEROXIDE RADICAL SCAVENGING ASSAY

This assay was based on the capacity of the Sangu chunnam to inhibit the photochemical reduction of Nitrobluetetrazolium (NBT) in the presence of the riboflavin-light-NBT system (Tripathi and Pandey Ekta, 1999; Tripathi and Sharma, 1999). Each 3 ml reaction solution contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM Ethylene diamine tetra acetic acid (EDTA), NBT (75 µM) and different concentration of extracts. It was kept visible in fluorescent light and absorbance was taken after 6 min at 560 nm by using a Deep Vision (1371) UV–Vis Spectrophotometer. Identical tubes with reaction mixture were kept in the dark served as blanks. The percentage inhibition of superoxide radical activity was measured by comparing the absorbance of the control with test sample solution.

% Super oxide radical scavenging capacity= $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of both plant extracts fraction.

NITRIC OXIDE RADICAL SCAVENGING ACTIVITY

Nitric oxide scavenging ability of sangu chunnam was measured according to the method described by Olabinri et al. (2010). 0.1 ml of sodium nitroprusside (10 mM) in phosphate buffer (0.2 M, pH 7.8) was mixed with different concentration of extracts and incubated at room temperature for 150 min. After treated period, 0.2 ml of Griess reagent (1% Sulfanilamide, 2% Phosphoric acid and 0.1% N-(1-Naphthyl) ethylene diamine dihydrochloride) was added. The absorbance of the experimental sample was read at 546 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard. The percentage of inhibition was calculated by following equation.

% Nitric oxide radical scavenging capacity= $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

METAL CHELATING ACTIVITY

Metal chelating capacity of Sangu chunam was measured according to Iihami et al., (2003). 1 ml of different concentrations of flavonoid rich fraction was added to 0.05 ml of 2 mM ferric chloride solution. The reaction was initiated by the addition of 0.2 ml of 5 mm Ferrozine and the mixture was shaken vigorously. After 10 min, the absorbance was measured at 562 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard. The % inhibition of ferrozoine-Fe2+complex was calculated by following equation.

% Inhibition of ferrozoine-Fe^{2+}complex = $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

RESULT AND DISCUSSION

ABTS ASSAY OF SANGU CHUNNAM

The radical scavenging ability was measured by ABTS assay as per given in table 1. The inhibition percentage of the ABTS radical activity was assessed on average and high free radical-scavenging values were found in sanguchunnam83.19% with EC_{50} value 15.24µl/ml. The pure ascorbic acid was lower activity (78.39 with EC_{50} value 17.23) (Table-8 and Fig-13). Nevertheless, in present study, it is showed that these activities were mainly due to nano minerals and hydroxyl compound present in Sangu chunnam. Antioxidants fight against free radicals and protect us from various chronic diseases, such as cardiovascular disease, atherosclerosis, cancer and the aging process (Sofia Rashid et al., 2016).

Table 1: Free radical-scavenging ability using ABTS assay of sanguchunnam

Different concentration of extract	Percentage of ABTS radicalactivity	
	Sangu Chunnam	Ascorbic acid (+ve control)
5 µl/ml	25.31±2.16	23.47±1.29
10 µl/ml	43.16±1.64	38.49±1.34
15 µl/ml	65.24±1.49	61.28±2.24
20 µl/ml	83.19±2.19	78.39±2.13
EC_{50} value	15.24	17.23

[a]Results are expressed as percentage inhibit of ABTS ability with respect to control. Each value represents the mean+SD of three experiments.

INHIBITION OF LIPID PEROXIDATION ACTIVITY OF SANGU CHUNNAM

The Sangu Chunnam also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was recorded in SanguChunnam76.23% with EC_{50} value 18.23 µl/ml and lowest inhibition percentage ascorbic acid 71.23% with EC_{50} 20.78(Table-2). As it is identified that lipid peroxidation is the net result of any free radical attack on membrane and other lipid components present in the system, the lipid peroxidation may be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid). In the present study, egg yolk was used as substrate for free radical mediated lipid peroxidation, which is a non-enzymatic method. Normally, the mechanism of Sangu Chunnam compounds for antioxidant activity includes neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals.

Table-2: Inhibition of lipid peroxidation activity of Sangu Chunnam.

Different concentration of extract	Lipid peroxidation inhibition percentage	
	SanguChunnam	Ascorbic acid (+ve control)
5 µl/ml	21.46±1.46	17.23±2.46
10 µl/ml	38.28±0.23	34.26±0.69
15 µl/ml	54.13±0.83	48.23±2.07
20 µl/ml	76.23±2.46	71.23±1.36
EC_{50} value	18.23	20.78

[a] Results are expressed as percentage inhibit of lipid peroxidation with respect to control. Each value represents the mean+SD of three experiments.

SUPEROXIDE SCAVENGING ASSAY OF SANGU CHUNNAM

Sangu Chunnam exhibited powerful scavenging activity for superoxide radicals in a concentration dependent process than positive control. Sangu Chunnam showed highest radical activity in the percentage of 72.56% with EC_{50} value 19.36 µl/ml when compared to positive control 68.23%with EC_{50} Value 21.36 µl/ml (Table-3). One of the standard methods to produce Superoxide radicals is through photochemical reduction of nitro blue tetrazolium (NBT) in the presence of a riboflavin-light-NBT system. These superoxide radicals are extremely toxic and may be produced either through xanthine activity or through mitochondrial reaction. Superoxide radicals are reasonably a weak oxidant may decompose to form stronger reactive oxidative species, such as singlet oxygen and hydroxyl radicals.

Table 3: Superoxide scavenging assay of Sangu Chunnam.

Different concentration of extract	Percentage of Superoxide scavenging activity	
	Sangu Chunnam	Ascorbic acid (+ve control)
5 µl/ml	21.63±0.25	17.23±1.24
10 µl/ml	35.49±2.16	31.46±0.25
15 µl/ml	53.19±2.32	48.23±1.46
20 µl/ml	72.56±2.15	68.23±0.49
EC_{50} value	19.36	21.36

[a]Results are expressed as percentage of Superoxide scavenging activity with respect to control. Each value represents the mean+SD of three

METAL CHELATING ACTIVITYOF SANGU CHUNNAM

The metal chelating property of Sangu Chunnam was displayed as per Table-4. The Sangu Chunnam was evaluated for their ability to compete with ferrozine for ferrous iron in the solution. In this evaluation, the Sangu Chunnam hindered the formation of ferrous and ferrozine complex, signifying that they have chelating activity and are capable of capturing ferrous iron before ferrozine. The Sangu Chunnam reduced the greenish blue color complex immediately and showed the highest chelating activity 73.69%With EC_{50} Value 20.12 µl/ml than positive control Vitamin-C 69.23% with EC_{50} value 20.89 µl/ml.

Table-4: Metal chelating activity of Sangu Chunnam.

Different concentration of extract	Percentage of Metal chelating activity	
	Sangu Chunnam	Ascorbic acid (+ve control)
5 µl/ml	20.16±0.98	16.58±1.67
10 µl/ml	34.56±2.89	30.26±2.49
15 µl/ml	52.23±1.69	47.25±0.25
20 µl/ml	73.69±2.46	69.23±1.98
EC_{50} value	20.12	20.89

Results are expressed as percentage of Metal chelating activity with respect to control. Each value represents the mean+SD of three experiments.

NITRIC OXIDE RADICAL SCAVENGING ASSAYOF SANGU CHUNNAM

Nitric oxide radical quenching activity of Sangu Chunnam was identified and compared with the standard ascorbic acid. The Sangu Chunnam displayed the maximum inhibition of84.23% at a concentration of 20µg/ml, in a concentration-dependent process when compared to ascorbic acid with inhibition percentage 78.23% (Table-5). In the current study, nitrite was produced by incubation of sodium nitroprusside in standard phosphate saline buffer at 25°C

was reduced by Sangu Chunnam. Significant scavenging activity may be due to the antioxidant property of flavonoid, which compete with oxygen to react with nitric oxide, leading to less production of nitric oxide.

Table-5: Nitric oxide radical scavenging assay of the Sangu Chunnam.

Different concentration of extract	Percentage of Nitric oxide radical scavenging activity	
	Sangu Chunnam	Ascorbic acid (+ve control)
5 µl/ml	24.16±0.81	21.45±2.18
10 µl/ml	46.23±3.19	41.26±1.67
15 µl/ml	63.21±2.16	59.35±1.69
20 µl/ml	84.23±1.69	78.23±2.49
EC_{50} value	14.63	16.45

[a] Results are expressed as percentage of Nitric oxide radical activity with respect to control. Each value represents the mean+SD of three experiments.

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