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Evaluation of in vitro antioxidant activity of Shilajitvadi Rasayana: A polyherbo-mineral Ayurvedic formulation

Research Article

Mayuri Dani[1*], Raman Belge[2]

1. PhD Scholar, Rasashastra & Bhaishajya Kalpana, Government Ayurveda College. Nagpur. India.
1. Associate Professor and HOD, Department of Rasashastra and Bhaishajya Kalpana, Swami Vivekanand Ayurved Medical College, Pargao, Shrigonda. Ahmednagar. India.
2. Professor & Head, Department of Rasshastra & B.K, Shri Ayurved Mahavidhyala, Nagpur. Maharashtra. India.

Abstract

Background: *Ayurveda* has a specialised branch to arrest/delay ageing and rejuvenate/revitalise the whole functional dynamics of the body system, known as the *'Rasayan chikitsa'* (Rejuvenation therapy). According to modern science, excess free radical production originating from endogenous or exogenous sources might play a role in many diseases. Antioxidants prevent free radical-induced tissue damage by preventing the formation of radicals, scavenging them, or promoting their decomposition. *Shilajitvadi Rasayana* (SHR) is a polyherbal-mineral formulation mentioned in *Rasaratnasamucchya* under *Rasayana chikitsa* to treat ageing and age-associated diseases. Being an Anti-ageing drug, SHR may have antioxidants and free radical scavenging activity to minimise free radical-induced damage, a key cause of ageing. Methods: The SHR was evaluated *in vitro* for its antioxidant activity using free radical scavenging activity, such as DPPH assay (2,2-diphenyl-1-picryl hydrazyl) and nitric oxide reducing assay. Result: In DPPH assay, the IC50 of SHR was 3584±144µg/ml, compared to (ascorbic acid standards) 0.191±0.005 µg/ml. In the Nitric oxide assay, the IC50 of SHR was 3150±370 µg/ml, as compared to Sodium Nitrate standards of 5.58±0.08 µg/ml. Conclusion: In both assays, SHR shows nearly the same IC50 value as 3584±144µg/ml and 3150±370 µg/ml, whereas the values of standards were 0.191±0.005 µg/ml and 5.58±0.08 µg/ml. Shilajitvadi Rasayana possesses good antioxidant activity, and the scavenging effect increases with the concentration of test compounds. The results of this study suggest the antioxidant and free radical scavenging activity of SHR. This might explain its *Rasayana effect* and justify its use as a medicine for age-associated diseases.

Keywords: *Shilajitvadi Rasayana, Ayurvedic A*nti-oxidant, Free radical scavenging, Anti-Ageing formulation, *Rasayana chikitsa*, Natural antioxidants.

Introduction

Oxidative stress is a significant factor in many diseases. Free radicals are highly reactive molecules that can damage cells, proteins, and DNA, leading to inflammation and health issues. Oxidative stress is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids, and deoxyribonucleic acid in healthy human cells and cause protein and deoxyribonucleic acid damage along with lipid peroxidation. These changes contribute to forming cancers, atherosclerosis, cardiovascular diseases, many other inflammatory diseases, and ageing. (1,2) All human cells protect themselves against free radical damage by enzymes such as superoxide dismutase and catalase, or compounds such as ascorbic acid, tocopherol, and glutathione. (3) Sometimes, these protective mechanisms are disrupted by various pathological processes. Hence, antioxidant supplements are vital to combat oxidative damage. A complex antioxidant system protects the human body by maintaining an antioxidant-prooxidant balance. Antioxidants delay or prevent free radical damage. (4) The antioxidant defence system consists of a network of enzymatic, non-enzymatic, endogenous and exogenous factors acting synergistically.

Recently, much attention has been directed toward developing “ethnic medicines” that possess potent antioxidant properties and are less toxic.

Rasayana tantra is a unique branch of *Ayurveda*, and the drugs mentioned in this chapter have been described to cure disease and promote health. (5) In general, *Rasayana* drugs promote healthy longevity, memory and intellect, preserve youthfulness, the lustre of the skin, and clarity of voice, and strengthen all organs in the body. (6) *Shilajitvadi Rasayana* (SHR) is a polyherbo-mineral compound, a traditional formulation from a classical text of *Rasaratnasamuchya,* which says SHR enhance health and supports the Dhatus within 15 days from intake.

*** Corresponding Author:**

Mayuri Dani

Associate Professor and HOD, Department of RSBK, Swami Vivekanand Ayurved Medical College, Pargao, Shrigonda and PhD Scholar, Government Ayurveda College, Nagpur. Maharashtra. India.

Email Id: mayuridani.md@gmail.com

Hence, this study was designed to provide a scientific basis for the action of *Rasayana* mentioned in the texts. This study aimed to evaluate the antioxidant potential and free radical scavenging activity of SHR in DPPH and Nitric oxide assays.

Materials and Methods

Drug Preparation

Shuddha Loha (Purified Iron) was subjected to *Bhavana* (Trituration of material with media) (7) with a freshly prepared decoction of *Triphala* (dried fruits of *Emblica officinalis, Terminalia bellirica and Terminalia chebula*) five times as per textual reference. (8) It took 21 *Gajaputas* (Measurement of Heat) to prepare the desired quality of (Passed all *Bhasma Pariksha*) *Loha Bhasma* (Incinerated Iron). *Shuddha Swarnmakshik* (Purified Copper Pyrite $CuFeS_2$) was triturated with freshly prepared *Nimbuk Swaras* (Lemon juice). (9) This process was supposed to be repeated ten times per the reference. [10] But *Swarnmakshik Bhasma* (Incinerated copper pyrite) was obtained after 22 *Gajaputa.* For the preparation of *Parad Bhasma* (Incinerated Mercuric Sulphide), *Kajjali* (Mercuric Sulphide HgS) was triturated in *Ankol mul twaka swaras* (juice prepared from the root bark of *Alangium Salvifolium*) and subjected to *Bhudhar puta.* (10)

To prepare SHR (11), the following ingredients were used: Shuddha *Shilajeet* (Purified *Asphaltum punjabium*), *Haritaki churna* (Powder of *Terminalia chebula*), *Vidang Churna* (Powder of *Embelia ribes*), *Loha Bhasma, Swarnmakshik Bhasma, Parad Bhasma*, 70 grams each of *Madhu* (Honey) and 70 ml of *Sarpi* (Clarified Butter). These components were combined in a mortar and pestle until a semisolid dough was formed. This mixture was then used to create a total of 940 handmade SHR pills, each approximately 500 mg in weight. Two pills were crushed and powdered and used as a sample for this study.

Evaluation of Solubility

For solubility evaluation, SHR was dissolved in the different organic solvent systems (with increasing polarity from 3.9 to 9) in 10mg/ml (stock) concentration, vortexed the mixture for 5 min and kept overnight at room temperature. Maximum solubility of SHR was found in DMSO (Dimethyl sulfoxide) (polarity index (PI) 7.8) as compared to water (PI-9), acetone (PI-5.1), methanol (PI-5.1), chloroform (PI-4.1), and isopropanol (PI-3.9).

Determination of Antioxidant Activity

DPPH radical scavenging activity

The DPPH assay was performed by the method described by Mohammad et al. (12) In brief, 4.3 mg of DPPH (1, 1–Diphenyl–2–picrylhydrazyl) was dissolved in 3.3 ml of methanol; it was protected from light by covering the test tubes with aluminium foil. 150 µl DPPH Solution was added to 3 ml of methanol, and absorbance was taken immediately at 517nm for the control reading. 50 µl of various concentrations of SHR and standard compound (Ascorbic acid) were taken, and the volume was adjusted to 150 µl using methanol. Each sample was diluted with methanol up to 3 ml, and 150 µl DPPH was added. Absorbance was taken after 15 min. At 517nm using methanol as a blank on a UV-visible spectrometer, Systronics India. The IC50 values for SHR and standard preparation were calculated. The DPPH free radical scavenging activity was calculated using the following formula:

% scavenging = [Absorbance of control - Absorbance of the test Sample/Absorbance of control] X 100

The effective concentration of the sample required to scavenge DPPH radical by 50% (IC50 value) was obtained by plotting a graph between %inhibition and concentration. (13)

Nitric oxide free radical scavenging activity

For the Nitric oxide assay, 500 µl of each of the concentrations of SHR and Sodium nitrate (standard compound) were taken separately. 2.0 ml of sodium nitroprusside (10 mm) in phosphate buffer saline was added to each tube. The solutions were incubated at room temperature for 150 minutes. A similar procedure was repeated with methanol as a blank, which served as a control. After the incubation, 3 ml of Griess reagent (Sigma) was added to each tube, including the control. The absorbance of the chromophore formed was measured at 546 nm on the UV-visible spectrometer Systronic India. Sodium nitrate was used as a positive control. The IC50 value for the test compound and standard preparation was calculated. (14-17)

% scavenging/Reduction = [Absorbance of control - Absorbance of the test Sample/Absorbance of control] X 100

Statistical analysis

All the statistical analysis was performed using MedCalc (version 10) using a t-test. P-values <0.05 were considered statistically significant.

Results

The antioxidant activity of SHR was tested by measuring their capacity to scavenge DPPH radicals. After plotting a graph of SHR in various concentrations (mg/ml) against % reduction in DPPH, it was found that absorbance increases when the concentration is increased in both SHR and Ascorbic acid (Standard). The IC50 value for *Shilajitvadi Rasayana* is 3584±144µg/ml, and the standard antioxidant Ascorbic acid showed an IC50 value of 0.191±0.005 µg/ml. (Table 1 and Figures 1 & 2). The tested sample reduced the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine.

As described previously, nitric oxide was generated from sodium nitroprusside and measured by the Griess reaction. Sodium nitroprusside in aqueous solution at physiological ph spontaneously generates nitric oxide (Green et al., 1982; Marcoci et al., 1994a, b), which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent. Nitric oxide scavengers compete with oxygen, producing reduced nitric oxide (Marcocci et al., 1994a, b). In the Nitric oxide free radical scavenging assay, SHR was

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compared with a standard Antioxidant, Sodium Nitrite (NaNo2). It shows that *Shilajitvadi Rasayana* possess good antioxidant activity, and the scavenging effect increases with the concentration of test compounds. The IC50 value for *Shilajitvadi Rasayana* is 3150± 370 µg/ml as compared to Sodium Nitrate standards of 5.58±0.08 µg/ml (Table 2 and Figures 3 and 4).

Table 1: Inhibitory concentrations (IC50 µg/ml) of *Shilajitvadi Rasayana* and its components by DPPH

Compound	Concentration	Absorbance	% reduction (% Scavenging)	IC50
Control (methanol)	0	0.217 ± 0.003	0	
Ascorbic acid (standard)* (in µg/ml)	0.1	0.178 ± 0.002	17.97235023	0.191 ±0.005 µg/ml
	0.2	0.091 ± 0.01	58.06451613	
	0.4	0.02 ± 0	90.78341014	
	0.8	0.014 ± 0.000	93.5483871	
	1	0.014 ± 0.001	93.5483871	
Shilajitvadi Rasayana (µg/ml)	1000	0.23 ± 0.01	0	3584± 144 µg/ml
	2000	0.182 ± 0.002	16.12903226	
	4000	0.087 ± 0.001	59.9078341	
	6000	0.032 ± 0.001	85.25345622	
	8000	0.031 ± 0.001	85.71428571	
	10	0.031 ± 0	85.71428571	

Figure 1: Free radical scavenging activity, % inhibition versus concentration for Standard Ascorbic acid. (original data)

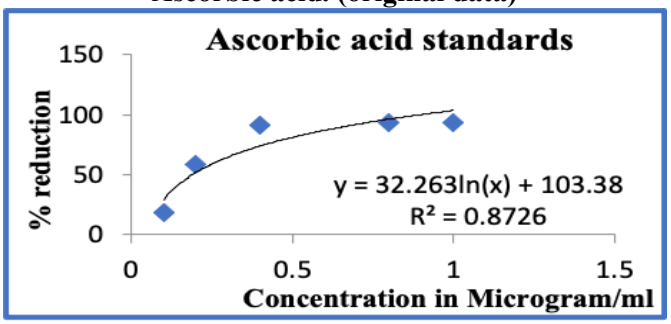


Figure 2: Free radical scavenging activity, % inhibition versus concentration for SHR (original data)

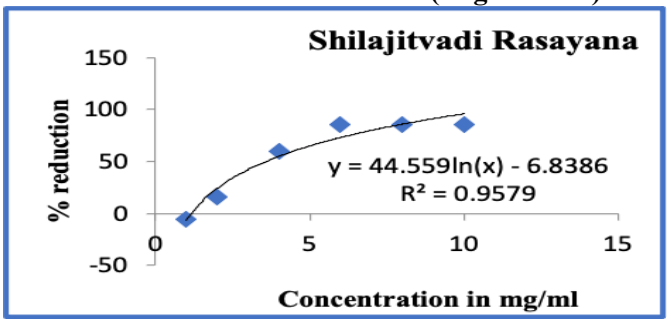


Table 2: Inhibitory concentrations (IC50 µg/ml) of *Shilajitvadi Rasayana* and its components by the Nitric oxide method

Compound Name	Concentration	OD (Mean±SD)	% reduction (% Scavenging)	IC50
Sodium Nitrite (NaNo2) Standards (in µg/ml)	Control	0.002 ± 0.000		
	10	0.112 ± 0.001	37	5.58± 0.08 µg/ml
	20	0.203 ± 0.001	67	
	30	0.258 ± 0.003	85	
	40	0.3 ± 0.001	99	
	50	0.334 ± 0.006	111	
Shilajitvadi Rasayana (µg/ml)	1000	0.072± 0.001	23	3150± 370 µg/ml
	2000	0.097 ± 0.001	31	
	3000	0.15 ± 0.030	49	
	4000	0.142 ± 0.002	47	
	5000	0.163± 0.003	54	

Figure 3: Free radical scavenging activity, % inhibition versus concentration for Sodium Nitrite. (original data)

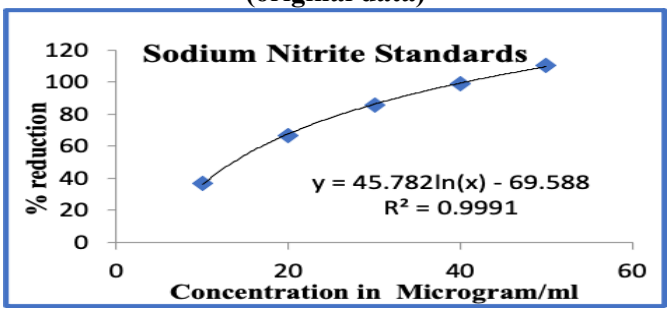
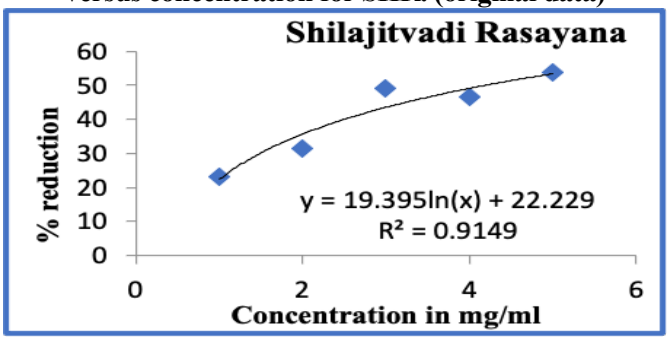


Figure 4: Free radical scavenging activity, % inhibition versus concentration for SHR. (original data)



Discussion

The numerous pathological events are associated with generating reactive oxygen species (ROS), constituting a key mechanism of tissue injury. They are relevant in inflammation, cardiovascular disease risk (18-19), and pathology of arteriosclerosis, malaria and

rheumatoid arthritis. They could play a role in neurodegenerative disease and premature ageing (20-22). Free radicals play an essential role in carcinogenesis through their involvement in breaking DNA strands (23). To address this problem, using natural antioxidants found in food supplements and traditional medicines like Rasayana has recently gained popularity. SHR is mentioned under Rasayana Chikitsa in Rasaratnasamuchya, a remedy known for its potential to counteract age-related degeneration. This study hypothesised that SHR, categorised as a Rasayana, might exhibit significant antioxidant activity.

The DPPH assay is a popular method to evaluate antioxidant activity because it is sensitive and straightforward. It is based on the theory that a hydrogen donor is an antioxidant. It measures compounds that are radical scavengers. DPPH is a well-known radical and a trap ("scavenger") for other radicals. Therefore, the rate reduction of a chemical reaction upon the addition of DPPH indicates the radical nature of that reaction. Nitric oxide is an essential bio-regulatory molecule required for several physiological processes like neural signal transmission, immune response, control of vasodilatation, control of blood pressure, etc. (24) However, the elevation of nitric oxide results in several pathological conditions, including cancer. NO is a short-lived (half-life 3–30s) colourless gas that is moderately soluble in water (up to 2 mmol/L) but highly soluble in organic solvents. (25) It is lipophilic and can diffuse between cells very easily. NO is generated from the terminal guanido nitrogen atom of L-arginine by various NADPH-dependent enzymes called NO synthases (NOS). (26) The three main isoforms are neuronal (n) NOS, inducible NOS, and endothelial (e) NOS. Generally, nNOS and eNOS are expressed constitutively in neurons and endothelial cells, respectively, though they can also be expressed by other cells. NO has an unpaired electron, hence it is a free radical (NO). NO becomes nitrosonium cation (NO^+) or nitroxyl anion (NO^-) by donating or accepting an electron, respectively. NOS is synthesised in various cell types from multiple mammalian species and can produce consistent, high concentrations of NO upon induction with cytokines and or bacterial lipopolysaccharide (LPS). (27)

While comparing SHR's antioxidant activity it reveals a few essential insights into its therapeutic potential. In the DPPH assay, SHR's IC50 value (3584±144 µg/ml) was significantly higher than that of ascorbic acid (0.191±0.005 µg/ml), indicating modest direct radical scavenging ability. However, this 18,000-fold difference must be contextualised—ascorbic acid is a pure, low-molecular-weight compound optimised for hydrogen donation, while SHR is a complex polyherbo-mineral formulation with diverse constituents.

More notably, in the NO scavenging assay, SHR demonstrated relatively better performance with an IC50 of 3150±0.370 µg/ml compared to sodium nitrite's 5.58±0.08 µg/ml. Though requiring a higher concentration than the reference standard, this 564-fold difference represents a narrower gap than observed in the DPPH assay. It suggests selective activity against physiologically relevant reactive nitrogen species. This difference in performance across both assays indicates that SHR possesses a different mechanism to exert its anti-oxidant activity, showing more potent activity against NO radicals than against DPPH radicals. This selective antioxidant profile is particularly significant given NO's role in inflammation and oxidative stress-related pathologies. The results suggest SHR may offer targeted protection against specific free radical species rather than functioning as a broad-spectrum antioxidant, aligning with traditional Rasayana principles of balanced, targeted biological activity. This selective mechanism may explain SHR's historical use in conditions now recognised to involve oxidative stress, despite its moderate potency in conventional antioxidant metrics.

Conclusion

In vitro antioxidant activity was carried out on *Shilajitvadi Rasayana* using the DPPH free radical scavenging and Nitric oxide methods. The IC50 value for the antioxidant activity of *Shilajitvadi Rasayana* was determined, which was nearly the same for both DPPH (3584±144µg/ml) and Nitric oxide assay (3150±370 µg/ml). Based on the results obtained in this study, it can be concluded that SHR exhibits substantial antioxidant and free radical scavenging activities. These in vitro assays suggest that this herbo-mineral combination is a significant source of natural antioxidants. Hence, *Shilajitvadi Rasayana* (SHR) could have great importance as a therapeutic agent in preventing diseases associated with oxidative stress. However, the components responsible for the antioxidant activity are currently unclear. Therefore, further investigation is needed to isolate and identify the antioxidant compounds present in the SHR extract.

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