Asian Journal of Medical and Pharmaceutical Sciences

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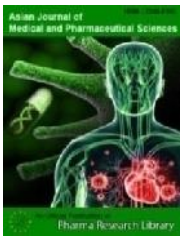

Research Article **Open Access**

Evaluation of Antioxidant Activity for Salvia Officinalis Using *In-Vivo* and *In-Vitro* Methods

Dr. Gampa Vijaya Kumar*[1], Dr. Y. Sridhar[2], B. Prathyusha[3], S. Raju[4]

[1]*Professor and Head, Department of Pharmacy, KGR Institute of Technology and Management, Rampally, Kesara, Rangareddy, Telangana, India.*
[2,3,4]*KGR Institute of Technology and Management, Rampally, Kesara, Rangareddy, Telangana, India.*

A B S T R A C T

On the basis of our findings, it may be worthy to suggest that *Salvia Officinalis* has antioxidant activity against Azathioprine induced oxidative stress in rats by decreasing the oxidative stress biomarkers serum creatinine, serum urea in kidneys.*Salvia Officinalis* has antioxidant effect, elevated by measuring antioxidant enzymes. There is increase in superoxide dismutase in liver and kidney tissues in Azathioprine induced oxidative stress in rats.*Salvia Officinalis* has high scavenging activity against DPPH free radical generating system.*Salvia Officinalis* has nephropretective effect against Azathioprine induced toxicity in kidneys by observing the histopathological changes in rat kidney tissues. *Salvia Officinalis* has many pharmacological activities like anticancer, antimicrobial, anti-inflammatory, analgesic, antiarthritic, antibacterial, anti-HIV, and antihelmntic activities.

Keywords: *Salvia Officinalis*, nephropretective, analgesic, antiarthritic, antibacterial, anti-HIV and antihelmntic activities

A R T I C L E I N F O

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Article History: Received 28 September 2016, **Accepted** 25 October 2016, **Available Online** 19 December 2016

***Corresponding Author**
Dr. Gampa Vijaya Kumar
Professor and Head, Dept. of Pharmacy,
KGR Institute of Technology and Management, Rampally, Kesara, Rangareddy, Telangana, India.
Manuscript ID: AJMPS3207

PAPER-QR CODE

Citation: Gampa Vijaya Kumar, *et al*. Evaluation of Antioxidant Activity for Salvia Officinalis Using In-Vivo and In-Vitro Methods. *A. J. Med. Pharm, Sci.*, 2016, 4(2): 65-70.

1. Introduction

Free radicals in Health and Disease:-A free radical is defined as any molecular species that contains an unpaired electron in the atomic orbital. Radicals are highly reactive that either donate an electron to or extract an electron from other molecules, and therefore, behave as oxidants or reductants. As a result of their high reactivity, most radicals have a very short half life (10-6 seconds or less) in biological systems. The most important free radicals produced in the body are oxygen derivatives, particularly superoxide and the hydroxyl radical. Examples of free radicals and reactive oxygen species include: superoxide anion radical, hydroxyl radical, nitric oxide, thiyl radical, trichloromethyl radical, hypochlorite radical, hypochlorous acid, and also some potentially dangerous non-radicals such as hydrogen peroxide, singlet oxygen, hypochlorous acid and ozone. Radical production in the body occurs by both endogenous and environmental factors. Since free radicals are causally involved in the disease state, it is believed that antioxidants should be effective in preventing or delaying their occurrence. Indeed, investigations at the cellular, tissue and whole animal level as well as epidemiological studies, strongly support the concept that nutritional antioxidant status is inversely related to the occurrence of free radical–mediated diseases

Antioxidants

An antioxidant is defined as: "any substance that, when present in low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate" (Halliwell, 1995). The physiological role of antioxidants is to prevent damage to cellular constituents arising as a consequence of chemical reactions involving free radicals.

An ideal antioxidant:

An ideal antioxidant should have the following attributes
No harmful physiological effects.

- Effective in low concentration. Fat-soluble.
- Carry-through effect.
- Not contribute an objectionable flavour, odour or colour to the food.
- No destruction during processing.
- Readily-available.
- Economical

Antioxidant Synergy:

Combination of antioxidants is more effective than the sum of the individual effects. Combined interaction improves effectiveness in several ways, for example: Vitamin E and C – Ascorbate can reduce Vit E, so in a lipid oxidation system Vit E and C together will be more effective than adding the effects of each alone. In biological samples synergy is also referred to as co - antioxidants. Antioxidant synergy is the key to the overall antioxidant defense system of living systems.

The antioxidant defense system

Since radicals have the capacity to react in an indiscriminate manner leading to damage to almost any cellular component, an extensive range of antioxidant defenses, have evolved to protect the cell from the free radical induced damage (Nordberg and Arner, 2001). The cellular antioxidants could be divided into three main groups: antioxidant enzymes, non-enzymatic antioxidants, and transition metal binding proteins.

2. Experimental

Methodology

Collection and Authentification of Plant Material

The Aerial Parts of *Salvia Officinalis* were collected and authenticated

Extraction of Plant Material

The plant is grinded in to a coarse powder with the help of suitable grinder.

Cold Extraction (Methanol Extraction)[38]

In this work the cold extraction process was done with the help of methanol. About 200gms of powdered material was taken in a clean, flat bottomed glass container and soaked in 750 ml of methanol. The container with its contents were sealed and kept for period of 7 days accompanied by continuous shaking with the shaker. The whole mixture then went under a coarse filtration by a piece of a clean, white cotton wool.

Evaporation of Solvent

The filtrates (methanol extract) obtained were evaporated using Rotary evaporator in a porcelain dish. They rendered a gummy concentrate of greenish black. The extract was kept in vaccum dissecator for 7 days.

% Yield value of Methanol Extract from Aerial Parts of *Salvia Officinalis* Plant

Powder taken for extraction = 200gm
Weight of the empty china dish = 53.0gm
Weight of the china dish with extract = 103.gm
Weight of the extract obtained = (103.0-53.0) gm = 50.0 gm
% yield of methanol extract = (weight of extract)/(powder taken for extraction) × 100 = 50/200 ×100 = 25.0 %.

***In-Vitro* Method:**

DPPH scavenging activity procedure: - DPPH radical scavenging activity was measured using the method of Cotelle et al., with some modifications. 3 ml of reaction mixture containing 0.2 ml of DPPH (100 µM in methanol) 2.8 ml of test solution, at various concentrations (5, 10, 20, 40, 80, 160 320 µg/ml) of the synthetic compound was incubated at 37°C for 30 min absorbance of the resulting solution was measured at 517 nm using Beckman model DU-40 spectrophotometer. The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control using the following equation:

% scavenging activity=absorbance of blank–absorbance of test / Absorbance of blank x 100

+ IC_{50} will obtain from a plot between concentration of test compounds and % scavenging. Ascorbic acid is used as standard for comparison.

***In-Vivo* Method:**

Experimental animals: 20 adult male albino rats weighing 140-160g were used for the study were procured. They were housed in polypropylene cages and were maintained at room temperature of $23^oC \pm 2^oC$ and relative humidity 50%. They were maintained in 12h: 12hr light: dark cycle throughout the period of acclimatization and experimental

study. Animals were provided with standard rodent pellet diet. Food and water was allowed *ad libitum.*

Acute toxicity study of *Salvia Officinalis* formulation (As per OECD guide Lines number: 423):

The Acute Toxicity Studies was performed using female rats as per OECD Guideline No.423 (short term toxicity). The compound found to be non toxic and safe up to 2000mg/kg body weight by oral route. After 48hr animals were well tolerated. There was no mortality and no signs of toxicity. So two doses are selected i.e. 100mg/kg and 200mg/kg are selected as low and high dose.

Induction procedure:

Induction of oxidative stress:

3mg/ml of Azathioprine solution was given through oral to all the group of animals and the samples were collected from the animals through retro-orbital plexus root and the kidney bio marker parameters were estimated like Creatinine, and Urea.

Collection of blood samples and organs:

Blood samples were collected from all the groups of animals 24hours after the 21st day of treatment through puncture of retro orbital plexus and were centrifuged at 3000 revolutions per minute (RPM) for 15 minutes. Serum was separated and stored at -20^{0}c and used for estimating urea and creatinine levels. Rats were killed by over anesthesia, a midline abdominal incision is made to open up the abdominal cavity and access the liver and kidney. The liver and right kidney are removed rapidly and washed with saline, then fixed quickly in formaldehyde. The liver and left kidney were homogenized in 0.25 M cold sucrose solution and centrifuged at 5000 rpm for five minutes. The supernatant which is store at -20^{0}C used for the quantitative estimation of superoxide dismutase within 48hours by using uv spectrophotometer [UV method].

Estimation of biochemical parameters:-

The following are the biochemical parameters estimated to evaluate the effect of the test materials against the experimentally induced oxidative stress in rats. They are SOD, Urea and Creatinine

Estimation of Superoxide Dismutase (SOD)

Superoxide dismutases are the enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. As such, they are an important antioxidant defense in nearly all cells exposed to oxygen. The enzyme superoxide dismutase (SOD) was determined in erythrocytes using photo oxidation method (Misra and Fridowich, 1977; Arutla *et al.,* 1998), which is briefly described below.

Principle:

In this assay free radicals are generated by photo-oxidation of o-dianisidine sensitized by riboflavin. The photo oxidation of O-dianisidine involves a complex series of free radical chain reactions involving the superoxide anion ($O_2^{\bullet-}$) as the propagating series (Figure 5.1). A general free radical scavenging compound has a inhibitory effect on this reaction leading to a decrease in the oxidized dianisidine measurable by UV/visible spectrophotometer. In contrast, any compound which specifically scavenges $O_2^{\bullet-}$ will remove the $O_2^{\bullet-}$ from step 3 and 4 in Figure 5.1 thus increasing the amount of oxidized dianisidine and hence will have an augmentary effect in this reaction. This assay can thus be used to determine whether a compound is a general, free radical or a scavenger specific for the super oxide anion. A substance with no free radical scavenging activity.

Reagents preparation:

For SOD estimation, 0.01M phosphate buffer (pH 7.5) was prepared.

Potassium phosphate buffer preparation:

Weight 1.741gms of K_2HPO_4, and dissolved in 1000ml distilled water, and 680.45mg of KH_2PO_4, and dissolved in 50ml individually, then the ph of K_2HPO_4 was observed. To K_2HPO_4, add aliquots of KH_2PO_4 until 7.5 pH attained which was measured with pH meter.

Preparation of riboflavin solution:

Riboflavin (5mg) was weighed, and dissolved in 1lit of potassium phosphate buffer, to attain concentration of $1.3*10^{-5}$ M.

Preparation of o-dianisidine solution:

O-dianisidine solution was prepared by weighing of 122mg and dissolved in 50ml of ethanol.

Extraction procedure: 3ml of packed blood cells were lysed by the addition of equal volume of cold deionized water. Hemoglobin was then precipitated by the addition of chloroform: ethanol (1.5:1). This was diluted with 500ml of water and centrifuged for 15 minutes at 3000 rpm. The supernatant containing SOD was taken for the measurement of its activity.

Assay procedure: 0.88ml of riboflavin solution (1.3×10^{-5} M in 0.01M potassium phosphate buffer, pH 7.5) was added to 60ml of O-dianisidine solution (10^{-2} M in ethanol) and to this 100ml of clear separated SOD was added and optical density was measured at 460nm. Then the cuvette containing reaction mixture was transferred to the illuminating box, illuminated for 4min., and optical density was remeasured against blank containing ethanol in place of enzyme. The change in the optical density was determined. The SOD content was determined from the standard graph prepared using pure bovine SOD.

Estimation of serum Creatinine levels

Clinical Significance:

Creatinine is the catabolic product of creatinine phosphate, which is used by the skeletal muscle. The daily production depends on the muscular mass and it is excreted out of the body entirely by the kidneys. Elevated levels are found in renal dysfunction, reduced renal blood flow and were decreased in muscular dystrophy.

Estimation of serum urea levels

Clinical significance: Urea is the major metabolite product of protein catabolism. The biosynthesis of urea from ammonia is exclusively carried out by hepatic enzymes. More than 90% of urea is excreted through the kidneys, with the remainder excreted through the gastrointestinal tract or skin. Blood urea concentrations can be increased by numerous factors linked to prerenal causes (increased protein catabolism, as in hemorrhage into gastrointestinal tract, shock, some chronic liver diseases) or renal/postrenal causes (acute or chronic renal diseases, post renal obstruction to urine flow). Uremia is also increased by high-protein diet, state of dehydratation, muscle wasting (as

in starvation). The determination of urea rate is used together with the determination of creatinine rate to discriminate between prerenal (normal creatinine) and renal /postrenal (increased creatinine) disorders.

Statistical analysis:

All the values were expressed as mean ±standard deviation (S.D). Statistical comparisons between different groups will be done by using one way analysis of variance (ANOVA) followed by dunnett's test. P <0.05 will be considered as statistically significant.

3. Results and Discussion

% Yield of the *Salvia Officinalis* is found to be **25.0**

Table 1: Concentration dependent percentage inhibition of DPPH radical by various concentrations of test compound and ascorbic acid

Concentrations of test compound and ascorbic acid (µg/ml)	Percentage inhibition of DPPH radical (IC_{50})	
	***Salvia Officinalis* (EESO)**	**Ascorbic acid**
5	17.3±0.71	47.6±0.48
10	25.2±0.31	56.15±0.65
20	31.3±1.0	65.6±0.48
40	38.4±0.7	70±1.33
80	40.7±0.35	77.8±0.82
160	47.6±0.7	84.9±1.1
320	57.7±0.5	89.1±0.51

The test compounds have been reported to show high scavenging activity against the DPPH free radical generating system. The antiradical activity of test compound and ascorbic acid against DPPH was shown in Table and the IC_{50} values were found to be as 17.3±0.71, to57.7±0.5 increased with respectively concentrations with that of reference standard, ascorbic acid (47.6±0.48 to 89.1±0.51).

The results clearly indicate the free radical scavenging activity of test compound in vitro and this activity comparable with that of standard drug ascorbic acid.

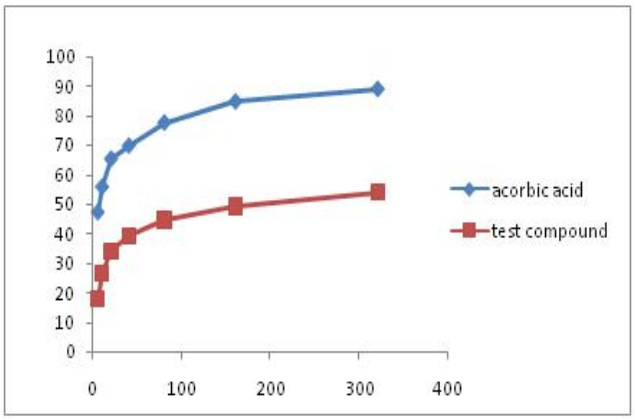

Figure 1: *In-vitro* concentration dependent percentage inhibition of DPPH radical by EESO and ascorbic acid

Table 1: Superoxide dismutase levels in kidney tissue homogenate

Group	SOD (U/mg) in kidney
Normal group	98.6±0.95
Toxic control (20mg/kg)	13.2±0.22
EESO low dose(100mg/kg)	39.7±0.6**
EESO high dose (30mg/kg)	57.6±1.1***
Standard ascorbic acid (10mg/kg)	80.2±0.84***

All the values are expressed as mean ±SD (n=6); ** indicates p<0.001, *** indicates p<0.0001 vs toxic control.

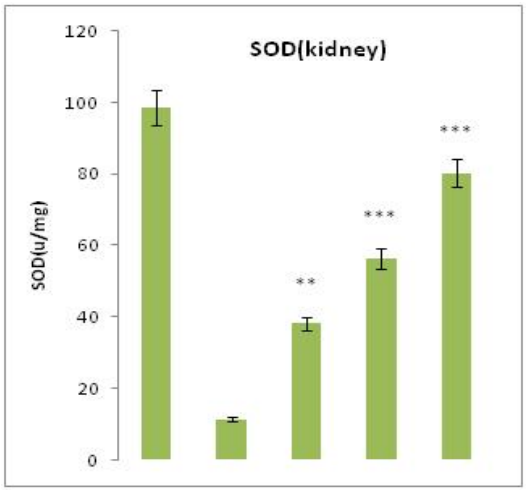

Figure 2: Effect of EESO on superoxide dismutase levels in kidney tissue homogenate in rats treated with AZP.

Table 2: Effects of EESO on serum creatinine levels in rats treated with azathioprine

Groups name	Creatinine (mg/dl)
Normal group	3.5± 0.32
Toxic control (20mg/kg)	24.2± 0.4
EESO low dose(100mg/kg)	6.1± 0.7***
EESO high dose (200mg/kg)	4.8± 0.3***
Standard ascorbic acid(10mg/kg)	4.3± 0.1***

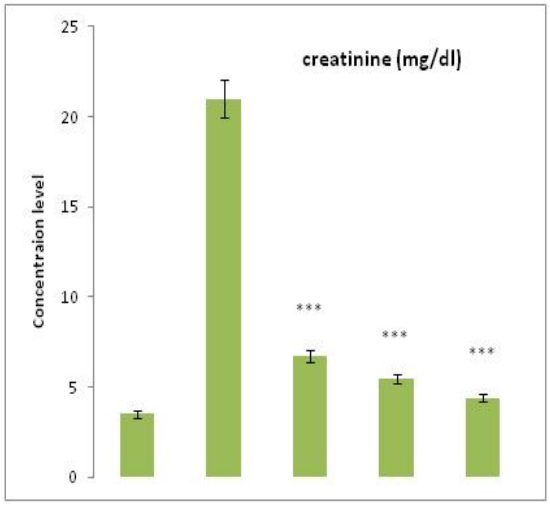

Figure 3: Effects of EESO on serum creatinine levels in rats treated with azathioprine

Table 3: Effects of EESO on serum urea levels in rats treated with azathioprine

Group name	Urea (mg/dl)
Normal group	28.4± 0.6
Toxic control (20mg/kg)	53.1± 0.5
EESO low dose(100mg/kg)	49.0± 0.3**
EESO high dose (200mg/kg)	35.3± 0.3***
Standard ascorbic acid (10mg/kg)	30.6± 0.8***

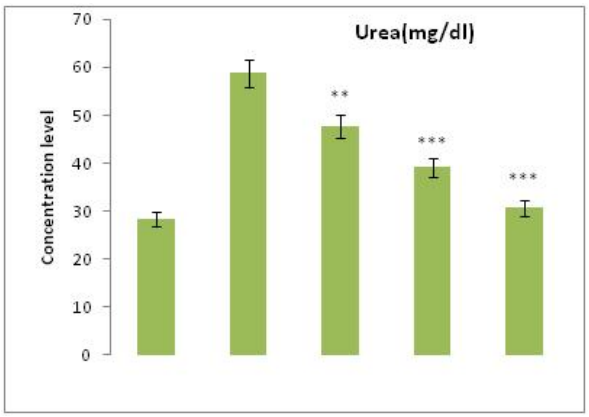

Figure 4: Effects of EESO on serum urea levels in rats treated with azathioprine

4. Conclusion

On the basis of our findings, it may be worthy to suggest that

- *Salvia Officinalis* has antioxidant activity against Azathioprine induced oxidative stress in rats by decreasing the oxidative stress biomarkers serum creatinine, serum urea in kidneys.
- *Salvia Officinalis* has antioxidant effect, elevated by measuring antioxidant enzymes. There is increase in superoxide dismutase in liver and kidney tissues in Azathioprine induced oxidative stress in rats.
- *Salvia Officinalis* has high scavenging activity against DPPH free radical generating system.
- *Salvia Officinalis* has nephropretective effect against Azathioprine induced toxicity in kidneys by observing the histopathological changes in rat kidney tissues.
- *Salvia Officinalis* has many pharmacological activities like anticancer, antimicrobial, anti-inflammatory, analgesic, antiarthritic, antibacterial, anti-HIV, and antihelmntic activities.

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