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Determination of SOD Activity of *Vayasthapana* Drugs *Guduchi Kwatha*, *Punarnava Kwatha* and *Punarnava* in Conjunction with *Guduchi Kwatha* in healthy Wistar Rats

Research Article

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Abstract

Superoxide dismutase (SOD) is an enzyme that serves as the primary defence against reactive oxygen species (ROS), playing a critical role in mitigating oxidative stress. *Guduchi* (*Tinospora cordifolia*) and *Punarnava* (*Boerhavia diffusa*), two herbs recognised in Ayurveda for their rejuvenating (*rasayana*) and anti-ageing (*vayasthapana*) properties, have been studied for their antioxidant potential. However, limited research exists on their combined effect on SOD activity in healthy subjects. This study aimed to investigate the individual and combined effects of *Guduchi* and *Punarnava* on SOD activity in healthy male Wistar rats. The animal experiment, approved by IAEC, included 32 rats divided into four groups: normal control (NC), trial group of *Guduchi* (TGG), trial group of *Punarnava* (TGP), and trial group of *Guduchi* + *Punarnava* (TGGP). For 30 days, distilled water (NC), *Guduchi kwath* (TGG), *Punarnava kwath* (TGP), and a combination of both (TGGP) were administered. SOD levels were measured before and after treatment using a spectrophotometric method. The study found statistically significant increases in SOD activity in TGG ($p = 0.001$), TGP ($p = 0.035$), and TGGP ($p = 0.010$), while the NC group showed no significant change ($p = 0.335$). These results suggest that *Guduchi*, *Punarnava*, and their combination enhance antioxidant defences, potentially slowing age-related changes and promoting healthier ageing.

Keywords: *Tinospora cordifolia, Boerhavia diffusa, Ayurveda,* SOD*, Vayasthapana, Rasayana, Kwatha,* Ageing.

Introduction

The free radical theory of ageing was proposed by Derham Harman (1). Theory suggests that reactive oxygen species (ROS) play a significant role in the ageing process by causing damage to cellular components such as DNA, proteins, and lipids. Harman later modified his theory in 1972 to emphasise the role of ROS produced in mitochondria, which is also called the powerhouse of energy (2). According to this modified theory, ROS produced within mitochondria can cause mutations in mitochondrial DNA, leading to further ROS production and accumulation of free radicals within cells. This mitochondrial theory of ageing has gained widespread acceptance and is considered to play a major role in contributing to the ageing process. Moreover, the free radical theory has been expanded further and includes not only ageing but also the diseases related to degenerative changes of ageing. Free radical damage has been linked to various disorders such as diabetes mellitus, cancer, atherosclerosis, and arthritis.

Understanding the role of free radicals and oxidative stress in ageing and age-related diseases, the development of antioxidant therapies aimed to reduce their effects. These therapies involve the use of antioxidants to neutralise free radicals and reduce oxidative damage, potentially slowing down the ageing process and delaying the onset of age-related degenerative changes (3). Antioxidants play a crucial role in mitigating and preventing harm caused by free radical reactions due to their unique capacity to donate electrons, effectively neutralising the radicals without forming new ones. Acting as reducing agents, antioxidants shield biological structures from oxidative damage by passivating them against free radicals. This proactive mechanism helps in minimising the harmful effects of oxidative stress on cells and tissues, contributing to overall health and well-being (4).

SOD (Superoxide dismutase) belongs to a class of enzymes across various life forms in all kingdoms (5). It stands as the primary defence against damage caused by reactive oxygen species (ROS). These enzymes catalyse the conversion of superoxide anion free radicals (O2-) into less harmful substances, molecular oxygen and hydrogen peroxide. By reducing the levels of free radicals SODs play a pivotal role in cellular protection. Based on metal cofactors present in

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their active sites, SODs lead to four distinct groups Copper-Zinc-SOD (Cu, Zn-SOD), Iron SOD (Fe-SOD), Manganese SOD (Mn-SOD), and Nickel SOD. These various forms of SODs are differentially distributed across biological species and lie within different subcellular compartments (6). Functioning as a crucial cellular antioxidant, SODs contributed significantly to cellular defense mechanisms.

Guduchi, Latin name *Tinospora cordifolia* (TC) (7). It is a robust, smooth-stemmed, deciduous *valli* belonging to the Menispermaceae family, renowned for its potential health advantages. In Sanskrit, it is referred to as *amrita,* signifying its ability to demonstrate longevity. In Ayurveda, *guduchi is* described *as rasayana* (rejuvenating) and *vayasthapana*(anti-ageing) drug (8). *Guduchi* has been also utilised to address an array of ailments, such as leprosy, fever, asthma, loss of appetite (anorexia), jaundice, gout, sinus infections, diabetes, chronic diarrhoea, and dysentery (9).

Punarnava Latin name *Boerhavia diffusa* (BD) member of the Nyctaginaceae family, is generally called a spreading hogweed (10), characterised by its perennial nature, low-lying growth, pink blossoms, and adhesive fruits. Widely referred to as *punarnava,* literary means to be renewed or rejuvenated in Sanskrit. It earns this name due to its recognised anti-ageing attributes. Various parts of the *punarnava* plant are rich in diverse bioactive compounds, making it *rasayana* in traditional medicine. Its exceptional properties, including immune-boosting capabilities, restoration of youthfulness, & enhancement of physical and mental health, are highly esteemed and utilised (11).

While numerous studies support the use of TC and BD for their antioxidant properties there is a gap in research regarding the study of antioxidant properties of *guduchi* and *punarnava* in normal healthy subjects and the combined effect of *punarnava* in conjunction with *guduchi*. To address this, an investigation is proposed to assess the SOD activity of *guduchi kwath* (GK), *punarnava kwath* (PK), and a combination of *guduchi and punarnava kwath* (GPK) using Pyrogallol auto-oxidation. The objective is to gain insight into the potential effects of these herbal formulations on SOD activity.

Methodology

Animal experiment study

The experiment was carried out with the conformity of the institutional animal ethics committee (IAEC) no. DMIMS (DU)/IAEC/2019-20-09 dated 14.01.2021. The study was carried out as per CPCSEA Guidelines for Laboratory Animal facility. Animal experiment was carried out at D.M.I.M.S. animal house. Antioxidant assay was performed in an authentic Laboratory. The experimental study was designed to assess a study on the antioxidant activity of test drugs GK, PK and GPK using Pyrogallol auto-oxidation for estimation of SOD in Wistar rats. 32 (24+8 considering 20% drop out) male Wistar rats of age group 18 months, weighing between 150-280 gm. were selected for the study. 18 months rat is considered 45 years of a human being (12). The sample size was determined with the help of the Resource Equation method (13). 8 (6+2)animals per group were selected dropping out 20% with a simple random sample method. The infected rats showing signs of infection during the study were excluded.

Animal experiment procedure

The 32 Wistar rats (laboratory rats of species *Rattus noregicus*) were divided into four different groups of 8 (6+2) animals in each. The study was carried out on 24 Wistar rats out of 32, with 2 rats in each group considered as potential dropouts. Six rats were selected and marked on different body parts: head, back, tail, head & back, back & tail, and head & tail, for identification during the study. In each group there was 6+2 rat GK, PK and GPK *were* given Orally with the help of a 2 ml syringe in a dose of 8.1 ml/kg in different groups. Group 1 normal control (NC) group of Wistar rats received distilled water for 30 consecutive days. Group 2 trial group of *guduchi* (TGG), Wistar rats received *guduchi kwath* (GK) for 30 consecutive days in dose 8.1 ml/kg. Group 3 trial group of *punarnava*(TGP), Wistar rats received *punarnava kwath* (PK) for 30 consecutive days in 8.1ml/kg. Group 4 trial group of *guduchi* and *punarnava* (TGGP), Wistar rats received *guduchi* and *punarnava kwath* (GPK) for 30 consecutive days in dose 8.1ml/kg. All the above groups were given once daily with their respective test drugs for 30 days. The first day before the administration of the test drug the blood sample was collected through orbital puncture of 24 Wistar rats for assessment SOD level. After 30 days of drug administration (the animal will become 19 months old) rats were given rest for one night and then a blood sample through orbital puncture was collected for SOD assessment. In this way, SOD level assessment is done before starting of study and after a one-month distance. The remaining animal returned to the animal house, so they could use this animal after the washout period.

Table 1: Group of animals and intervention

Grp. No.	Group Name	Age	Grp. Code	Sample Size	Inter-vention	Route
1	Normal control	18 months	NC	6	Distilled water	Oral
2	Trial group *guduchi*	18 months	TGG	6	*Guduchi kwath*	Oral
3	Trial group *punarnava*	18 months	TGP	6	*Punarnava kwath*	Oral
4	Trial group *guiduchi* and *punarnava*	18 months	TGGP	6	*Guduchi* and *Punarnava kwath*	Oral

SOD activity assay

It is one of the body's foremost authenticate assays reducing free radical damage associated with many diseases. SOD converts superoxide radicles into hydrogen peroxide and molecular oxygen. Hydrogen peroxide is converted to water and O_2 with the help of catalysing. SOD activity assay measures superoxide dismutase activity using a standard suitable for use with plasma, serum, cell, or tissue (14). Higher SOD activity values have several important implications, especially regarding antioxidant capacity and cellular protection against oxidative stress (15). The instruments and equipment required for the study are a spectrophotometer and pH Meter. SOD assay performed with the help of chemical reagents as given in (Table 2).

Table 2: Chemicals and reagents used for the study

Sr. No.	Name of the Chemicals/Reagents Manufacturer	Manufacturer
1	Tris HCL buffer solution with Immol/L EDTA at pH 8.2	Sigma Aldrich Chemicals Pvt Ltd Industry
2	Pyrogallol Solution Cat no. 254002	Sigma Aldrich Chemicals Pvt Ltd Industry
3	Distilled Water	In House
4	Superoxide Dismutase Cas no. 9054-89-1	Sigma Aldrich Chemicals Pvt Ltd Industry

Superoxide Dismutase (SOD) activity assay procedure: (Cu-Zn) SOD activity was determined by using a simple and rapid method, based on the ability of the enzyme to inhibit to autoxidation of pyrogallol. The auto-oxidation of pyrogallol in the presence of EDTA at pH 8.2 is 50%. The principle of this method is based on the competition between the pyrogallol auto-oxidation by O2 and the dismutation of this radical by SOD. (Cu-Zn) SOD activities were demonstrated as units/ml. One unit of (Cu-Zn) SOD activity was defined as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation.

The UV-Visible Double Beam Spectrophotometer was calibrated to read zero using Tris EDTA buffer. Control and sample test solutions were prepared and pipetted into respective test tubes. (16 17). The absorbance was measured at a wavelength of 420 nm against the Tris EDTA buffer as a blank. Readings were taken initially (zero time) and then again after 1 minute following the addition of pyrogallol to assess the change in absorbance over time.

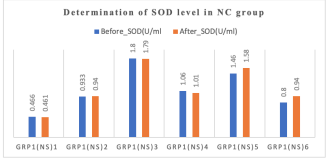
Safety precautions: Gloves, caps, face masks and goggles were used in addition to protective body garments and shoes to ensure adequate personal health, and safety and to avoid inhalation and Skin contact with the test item or toxic chemicals. In case of eye contact, the eye will be washed thoroughly with water and medical treatment will be sought. In case of skin contact, it will be washed with soap and water with subsequent medical aid.

Observations & Results

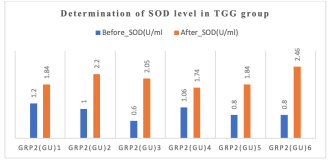
Table 3: Determination of SOD (U/ml) level before and after intervention of test drugs in NC, TGG, TGP, TGGP groups

NC Group	Before SOD(U/ml	After SOD(U/ml)
GRP1(NS)1	0.466	0.461
GRP1(NS)2	0.933	0.94
GRP1(NS)3	1.8	1.79
GRP1(NS)4	1.06	1.01
GRP1(NS)5	1.46	1.58
GRP1(NS)6	0.8	0.94
TGG Group	Before SOD(U/ml	After SOD(U/ml)
GRP2(GU)1	1.2	1.84
GRP2(GU)2	1	2.2
GRP2(GU)3	0.6	2.05
GRP2(GU)4	1.06	1.74
GRP2(GU)5	0.8	1.84
GRP2(GU)6	0.8	2.46
TGP Group	Before SOD(U/ml	After SOD(U/ml)
GRP3(PU)1	1.2	1.79
GRP3(PU)2	1.6	1.89
GRP3(PU)3	1.86	1.89
GRP3(PU)4	1.8	2.1
GRP3(PU)5	0.46	1.43
GRP3(PU)6	0.6	2
TGGP Group	Before SOD(U/ml	After SOD(U/ml)
GRP4(GU+PU)1	0.73	2.15
GRP4(GU+PU)2	1.2	1.43
GRP4(GU+PU)3	1.4	1.94
GRP4(GU+PU)4	0.86	1.89
GRP4(GU+PU)5	1.53	1.89
GRP4(GU+PU)6	1.33	2.35

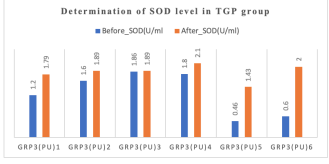
Graph 1: SOD levels before and after intervention in the NC group



Graph 2: SOD levels before and after intervention in the TGG group



Graph 3: SOD levels before and after intervention in the TGP group



Graph 4: SOD levels before and after intervention in the TGGP group

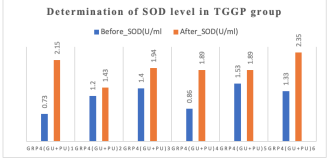


Table 4: Indicating mean, standard deviation and p- value of study groups

Group	SOD level U/ml	N	Mean	St. deviation	Sig(2 tailed)
NC	Before	6	1.0865	0.47748	0.335
	After	6	1.1202	0.48408	
TG	Before	6	0.09100	0.21679	0.001
	After	6	2.0217	0.27206	
TGP	Before	6	1.2533	0.60764	0.035
	After	6	1.8500	0.23160	
TGGP	Before	6	1.1750	0.3157	0.010
	After	6	1.9417	0.30896	

The statistical test used here is the Paired Sample t-Test (Dependent t-Test). This test is designed to compare the means of two related groups, such as measurements taken from the same subjects before and after an intervention. The NC group shows p-value 0.335, which is higher than the significance threshold of 0.05, indicating that there is not a statistically significant difference in SOD levels before and after intervention in the NC group. (Graph 1, Table 3 & 4) There is a statistically significant difference in SOD levels before and after intervention in the TG group as the p-value is 0.001 which is less than 0.05. (Graph 2, Table 3 & 4) The p-value is 0.035 which is less than threshold of 0.05, it suggests a statistically significant difference in SOD levels before and after intervention in the TGP group. (Graph 3, Table 3 & 4) The p-value is 0.010, indicating a statistically significant difference in SOD levels before and after intervention in the TGGP group. (Graph 4, Table 3 & 4) The TG, TGP, and TGGP groups statistically significant changes in SOD levels after giving GK, PK, and GPK intervention while the NC group did not show a significant difference.

Discussion

Oxidative stress is a major contributor to ageing processes at the cellular level. By reducing oxidative damage, higher SOD activity can potentially slow down the ageing of cells and tissues. In a broader health context, higher SOD activity is associated with improved overall health and reduced risk of certain diseases. Oxidative stress, characterised by an imbalance between reactive oxygen species (ROS) production and antioxidant defence can lead to cellular damage and contribute to various health issues, including ageing, inflammation, and chronic diseases. Because of the health-promoting potential of GK, PK and GPK to rule out SOD activity in Wistar rats study was planned. In the normal control group, SOD activity was observed non-significantly different as the p-value is 0.335. This means there is no reduction in oxidative stress and ultimately no effect on degenerative changes of ageing in the NC group after the one-month interventional period.

In the group that received GK intervention, there was a noteworthy increase in SOD levels before and after the treatment. Higher SOD activity indicates a more robust antioxidant defence mechanism within cells. This means the cells are better equipped to neutralise superoxide radicals, reducing oxidative damage to cellular components like DNA, proteins, and lipids. This may be occurring due to *rasayana* effect of *guduchi. Rasayana*, an integral facet of Ayurveda, concentrates on rejuvenation therapy, which carries deep significance in traditional medicine. *Rasayana* formulations not only strive to rejuvenate the body but also aim to enrich cognitive functions, fortify physical resilience, extend lifespan, and prevent illnesses. This holistic approach underscores the wide-ranging advantages of *rasayana* in fostering overall health and longevity, *guduchi* is one of the potent *Rasayana* drugs. study shows that *guduchi churna,* increases in the life span of the F-1 generation of *Drosophila* melanogaster (18). TC shows various chemical constituents such as alkaloids, glycosides, steroids, etc. which show potential in treating various ailments (19). One review also referred to the use of *guduchi* as *rasayana* can limit the anatomical and physiological changes of ageing (20).

In the TGP group, the p-value of 0.035 is suggestive of a significant effect on SOD levels after giving the intervention PK. High SOD activity means more effective neutralisation of superoxide radicals, reducing oxidative damage to cellular components such as proteins, lipids, and DNA. This protection is vital for maintaining cellular integrity and functionality, contributing to overall health and longevity. This suggests that the drug *punarnava* can avoid oxidative damage caused to cells due to free radicals and hence can avoid untimely ageing. *Punarnava*'s roots and shoots are packed with a diverse array of compounds like phenolics, rotenoids, flavonoids, isoflavonoids, alkaloids, steroids, anthracenes, and lignans, along with various proteins and fatty acids. This rich biochemical composition contributes to its remarkable therapeutic potential.[11] *Punarnava* is celebrated in India for its ability to address a multitude of health issues, including epilepsy, dysentery, diarrhoea, urinary and kidney problems, jaundice, anaemia, pneumonia, and splenomegaly. Its regular use is believed to rejuvenate the body, enhancing vitality, which aligns with its Sanskrit name, *punarnava*. It contains antioxidants that scavenge free radicals and increase glutathione content, aiding in the treatment of hepatitis and hepatic cirrhosis (21). *Punarnava is* a hepatoprotective adrenal protective drug as it has diuretic, anti-inflammatory, and immunomodulatory effects. *Punarnava* shows promise in treating a wide range of ailments. Considering its therapeutic potential.

The p-value is 0.010, indicating a statistically significant difference in SOD levels before and after

intervention in the TGGP. SOD is a crucial antioxidant enzyme that plays a central role in neutralising superoxide radicals, which are highly reactive and damaging to cells. Two drugs *guduchi* and *punarnava* in combination also show the potential of neutralising superoxide radicals which ultimately leads to age-related changes.

Although we couldn't determine exactly how GK, PK and GPK enhance survivability in this study, we did confirm its *vayasthapana* effect, which means its ability to support vitality and longevity. This was observed not only with *guduchi kwatha* alone but also with *punarnava kwatha* in combination with both (GPK). Charakacharya mentioned that out of ten drugs of *vayasthapana mahakashaya* any two or more drugs in different combinations can be used as per requirement (22). thus the combination of *guduchi* and *punarnava* also shows a significant effect on SOD activity. When interpreting these findings, it's crucial to consider the limitation of the study that various factors could influence the results, such as the composition of the samples, how they were prepared, the conditions of the experiments, and any additional substances or factors that might affect the activity of superoxide dismutase. In the future, research can propose to elucidate the pathways responsible for enhanced SOD activity. Clinical trials to assess the efficacy of interventions targeting SOD in human health or clinical trials aimed at evaluating the efficacy of antioxidants in disease prevention or treatment.

Conclusion

The conclusion drawn from this analysis is that SOD activity values in groups TGG, TGP and TGGP indicate more effective inhibition of superoxide radicals, showing more potential in terms of combating oxidative stress that may be caused due to degenerative changes of ageing. GK, PK and GPK protect against oxidative damage which promotes antioxidant defence mechanisms and thus supports the potentially slowing down age-related changes and promoting healthier ageing.

Conflict of interest: Authors declared no conflict of interest.

Abbreviations: ROS - reactive oxygen species, SOD - Superoxide dismutase, TC - Tinospora cordifolia, BD - Boerhavia diffusa, GK -*Guduchi kwath,* PK- *Punarnava kwath,* GPK – *Guduchi*, *Punarnava kwath,* NC - Normal control, TGG - Trial group *guduchi,* TGP -Trial group *punarnava,* TGGP Trial group *guiduchi* and *punarnava,* GU - *Guduchi*, PU - *Punarnava*, GUPU - *Guduchi* and *Punarnava,* GRP – Group.

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