RESEARCH ARTICLE

Appraisal of Antioxidant Potential of *Saussurea lappa* and *Valeriana wallichii* Root Extract by *In-vitro* Techniques

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ABSTRACT

Background: Antioxidants are very important in preventing and protecting from oxidative stress and, ultimately, oxidative stress-related disorders. With the aim to the antioxidant activity of *Saussurea lappa* and *Valeriana wallichii* extract, individually and in combination, the study was performed via assays for antioxidant capability; 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), nitric oxide scavenging method, ferric reducing antioxidant power (FRAP), H_2O_2 scavenging potential, superoxide radical scavenging method showed good antioxidant activity. Individually, *V. wallichii* having more superior antioxidant activity than *S. lappa*.

Results: Phytochemical analysis of both extracts individually illustrate the existence of anthraquinones, cardiac glycoside, flavonoids, reducing sugars, phenolics, saponins, alkaloid terpenoids, and phlobatannin. In combination, the antioxidant activity was improved, indicating additive effects. The results indicate that these herbs can be a very good constituent for combination therapy for diseases that are caused by oxidative stress and require antioxidant therapy. The *S. lappa* and *V. wallichii* showed average TPC of 68.38 and 117.52 mg GAE/g of extract, respectively. The mix of 1:1 ratio of both extracts showed TPC of 101.34 mg GAE/g. The *S. lappa* and *V. wallichii* showed average TFC of 59.72 and73.56 mg QE/g of extract, respectively. The mix of 1:1 ratio of both extracts showed a TFC of 66.15 mg QE/g of extract. Results showed that both extracts have very strong antioxidant activity. *V. wallichii* has relatively higher antioxidant activity and is close to that of ascorbic acid.

Keywords: Antioxidant, DPPH, FRAP, H_2O_2, *Saussurea lappa*, Superoxide radicals, *Valeriana wallichii*.

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INTRODUCTION

Since ancient times, medicinal plants have been used to treat a variety of diseases. About three fourth of the population is still dependent on plants for medicines.[1] Being the origin of the Ayurvedic system, India is home to many medicinal plants.[2,3] Herbal drugs are generally considered safe drugs with less toxic effects.

The pharmaceutical industry and researchers are showing enhanced curiosity in plant-based drugs compared to synthetic drugs, for which cost and safety are the main concerns.

Oxidative stress is a condition caused by free radicals, which are entities with unpaired electrons. Reactive oxygen species (ROS) are also responsible for oxidative stress and are generated internally through aerobic cellular respiration and externally by pollution and ionizing radiation. Both these are directly or indirectly responsible for many metabolic diseases like gastric ulcers, cancer, diabetes, and atherosclerosis, including nervous system disorders.[4-7] Living organisms defend themselves from such harm by either endogenous antioxidant defense systems or by taking dietary antioxidants. Enhancing the body's natural antioxidant defenses or providing supplementary dietary antioxidants can prevent the risk of the occurrence of many chronic diseases and their progression.[8] Antioxidants exert their effect in several ways, like preventing the formation of free radicals or interrupting and disturbing oxidizing chain reaction.[9]

Researchers are focusing on natural antioxidant agents which trap potentially harmful free radicals for the protective effects against oxidative stress.[10] With the development of recent medicinal technology, it is easier to recognize specific botanical chemical constituents and evaluate their potential antioxidant activity. The antioxidants of natural or herbal origin are safe and good for health and can be used for oxidative stress management.[11]

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Saussurea lappa belonging to family asteraceae, has medicinally huge potential to treat complicated diseases like anticancer/antitumor,[12-15] hepatoprotective,[16] anti-ulcer and cholagogi,[16-19] immunomodulator,[20] anticonvulsant activity,[21] gastric functions,[16] gastro-protective effect.[22] angiogenesis effect,[23] hypoglycaemic,[24] antiviral activity, spasmolytic activity.[25] and antiparasitic.[26-28] *Valeriana wallichi* belonging to the family valereanaceae, is another herb that is traditionally known to use in a variety of diseases, including analgesic,[29] and anti-inflammatory,[30] myorelaxant and antispasmodic,[29] psychotic activity.[31-34] radioprotective activity, antimicrobial activity.[35]

There is a need to explore the potential of these herbs to contest life-threatening diseases, especially if they show increased therapeutic effects, a reduced profile of side effects, or either both.[36-38] The extracts of these drugs are evaluated in combination for the antioxidant effect to determine if they show an enhanced effect.

MATERIALS

Plant Material and Extract Preparation

Herbal drugs were procured from Indian Druggist Tirupati, India. They were authenticated before use from Department of Botany, SV University, Tirupathi. The medications were maintained in an airtight container in cool, dry location after being dried in the shade and crushed into a coarse powder. The remaining other reagents used were of AR grade.

METHODS

Preparation of Extract

Both drugs were extracted individually in a soxhlet extractor with ethanol: water mixture in 1:1 ratio at a temperature not more than 80°C. The semisolid extract was dried to get a solid mass, labeled, and stored.

Preliminary Phytochemical Screening

The preliminary phytochemical screening was carried out for both extracts for the detection of various phytochemicals.

Estimation of Total Phenolic Content (TPC)

By using the Folin-ciocalteu technique, the TPC of extracts was calculated.[39] Sample extract (0.3 mL) combined with 1.2 mL of 7.5% w/v Na_2CO_3, 1.5 mL of 10% v/v Folin-reagent, Ciocalteu's and 30 minutes of dark incubation before being measured for absorbance at 765 nm using a UV spectrophotometer (Schimadzu 1800). Gallic acid equivalents were calculated as mg per 100 g of extract as the outcome**.**

Estimation of Total Flavonoid Content (TFC)

By using quercetin as a reference, an aluminum chloride complex assay was utilized to assess TFC.[40] 100 µL of each of the produced quercetin solutions- 0.1, 0.5, 1.0, 2.5, and 5 mg/mL in methanol- were combined with 500 µL of distilled H_2O_2 and 100 µL of 5% sodium nitrate separately. 150 µL of a 10% aluminum chloride solution were added after around 6 minutes and maintained for 5 minutes. After recording the absorbance at 510 nm using a UV spectrophotometer, 200 µL of a 1M sodium hydroxide solution was combined, and a calibration curve was constructed. The samples were examined using the same process, and TFC was determined as mgQE/g of quercetin.

Evaluation of *In-vitro* Antioxidant Activity

DPPH Scavenging Assay

DPPH free radical scavenging was determined to evaluate antioxidant activity[41] using ascorbic acid as standard. DPPH solution (1-mL) in methanol (0.1 mM) was added to 1-mL solution of extracts in methanol prepared in various concentrations of 5–30 µg/mL and maintained for 30 minutes at room temp in the dark and analyzed spectrophotometrically at 517 nm, and scavenging activity was estimated.

Hydroxyl Radical Scavenging Method[42]

100 µL of hydrogen peroxide (1 mM), 100 µL of deoxyribose (2.8 mM), 200 µL EDTA (0.1 mM), 200 µL of ferric chloride (0.1 mM), and 100 µL ascorbic acid (0.1 mM) were added to phosphate buffer at pH 7.4 (20 mM) (0.1 mM). Different extract concentrations (500 µL) were separately added to the reaction mixture and incubated for 1 hour at 37ºC. At last, each reaction mixture received 1.0 mL of tricholoacetic acid (2.8%) and 1.0 mL of thiobarbituric acid (1%) before being heated in a water bath for 20 minutes. Similar preparations were made for the control and blank. Using a UV spectrophotometer at 532 nm, the absorbance of each sample was considered against a blank. The absorbance readings of the sample and the control were used to compute %inhibition.

Nitric Oxide Scavenging Method

Different amounts of extracts (0.2–0.8 mg/mL) were combined with 0.5 mL of saline phosphate buffered (pH 7.4) and 0.5 ml of sodium nitroprusside (10 mM) and incubated at 30ºC for 2.5 hours before adding 1.0 mL of Gries reagent.[43] By measuring absorbance at 548 nm against a blank surface, a UV spectrophotometer was used. By comparing the absorbance of an extract with control, %inhibition of nitric oxide production was resolute.

Ferric Reducing Antioxidant Power (FRAP Assay)

Acetate buffer (pH 3.6), 2, 4, 6-tripyridyl-s-triazine (10 mM), and ferric chloride solution (20 mM) were combined to create a fresh working FRAP solution, which was then maintained at 37°C. Extracts were added, and 2.8 mL FRAP solution was permitted to react for 30 minutes in the dark.[44] By using UV, absorbance at 593 nm was recorded. Results were given as g Fe (II)/g dry mass.

Superoxide Radical Scavenging Method

Non-enzymatic phenazine methoxy sulfate-nicotinamide adenine dinucleotide was used to make superoxide anions, which were then produced and measured by reducing nitro blue tetrazolium (NBT).[45] In order to create solutions with various extract concentrations, phosphate buffer, pH 7.4, 1 mL of NBT (50 µM) solution, and 1 mL of NADH (78 µM) solution were all added. A 1-mL of PMS solution (60 µM) was added to

Table 1: Phytochemical analysis of extracts of *S. lappa* and *V. wallichii*

Constituents	*S. lappa*	*V. wallichii*
Flavoinds	++	+++
Phenols and Tannins	++	++
Sapnoins	+	-----
Anthraquinones	+	-----
Cardiac glycosides	+	+
Terpenoids	+	+
Alkaloids	+	+
Steroids	----	---
Phlobatannins	+	---
Quinones	---	+

(+) indicated presence and (–) indicated absence

mixtures to begin the reaction, which was then incubated for 5 minutes at 25°C. Utilizing UV, absorbance at 560 nm was measured in comparison to blank.

RESULTS

Extract Yield

Accurately weighed drug powder was treated with 50% ethanol and 50% water, and Soxhlet apparatus was used for extraction. The extract was concentrated under a vacuum, dried in vacuum desiccators (yield 23% w/w), and then kept in the refrigerator for further use.

Phytochemical Analysis

Different phytochemicals were detected in all the extracts, and results are tabulated as follows.

Total phenolic content (TPC) of extracts of *S. lappa* and *V. wallichii* individually and in combination are presented in Figure 1. Results were designed as mg GAE/g of extract using a gallic acid standard curve. The *S. lappa* and *V.wallichii* showed average TPC of 68.38 and 117.52 mg GAE/g of extract. The mix of 1:1 ratio of both extracts showed TPC of 101.34. It is hypothesized that the phenolic molecules' redox characteristics account for their antioxidant effects. Free radicals are neutralized and blocked by the redox potential, singlet and triplet oxygen are neutralized, and peroxides are broken down.[46]

The total flavonoid content (TFC) of extracts of *S. lappa* and *V. wallichii* individually and in combination are presented in Figure 1. Outcomes were determined as mg QE/g extract by using the quercetin standard curve. The *S. lappa* and *S. lappa* showed average TFC of 59.72 and73.56 mg QE/g of extract, respectively. The mix of 1:1 ratio of both extracts showed a TPC of 66.15 mg QE/g of extract. The flavonoid compounds have scavenging or chelating potential, which may be responsible for their potent antioxidant activity as they can deactivate highly active oxidative moieties by this mechanism.[47]

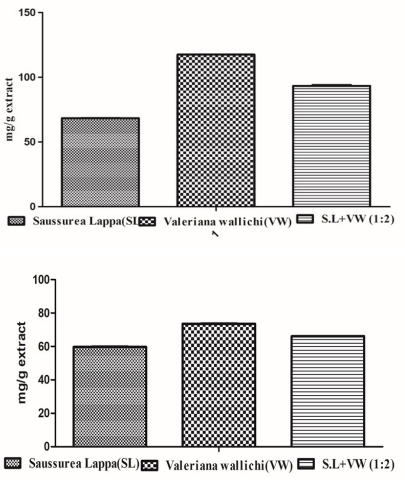

Figure 1: (A) Total flavonoid contents and (B) Total phenolic contents

DPPH Free Radical Scavenging

The extracts of *S. lappa* and *V. wallichii* (5.0 to 30.0 mg/mL) were estimated for their antioxidant perspective, individually and in combination, by *the in-vitro* DPPH free radical scavenging method. *S. lappa* extract showed 71.23 % scavenging, *V. wallichii* showed 82.72%, 1:1 ratio of both extracts had 74.53%, 1:2 ratio of both extracts had 80.66 and 2:1 ratio of both extracts had 73.48% activity at 30.0 mg/mL. At the same concentration ascorbic acid exhibited 91.89% scavenging. The results are depicted in Figure 2. *V. wallichii* showed the highest activity among the tested samples. The activity was concentration-dependent and showed a linear increase with concentration. The studied extracts' high levels of TPC and TFC and strong proton-donating capacity may cause their antioxidant activity.

Hydroxyl Radical Scavenging

Ascorbic acid was used as a reference standard to assess the hydroxyl radical scavenging activity of the extracts both singly and in combination at varying concentration levels, varying from 5.0 to 30.0 mg/mL. Activity increased with an increase in concentration levels (Figure 3). *S. lappa* extract showed 73.23% scavenging, *V. wallichii* showed 89.63%, 1:1 ratio of both extracts showed 78.92%, 1:2 ratio of both extracts showed 81.66% and 2:1 ratio of both extracts showed 75.48% at 30.0 mg/mL. Ascorbic acid at the same concentration exhibited 99.89% scavenging. The positive correlation between concentration and activity indicating activity increases with increased concentration.

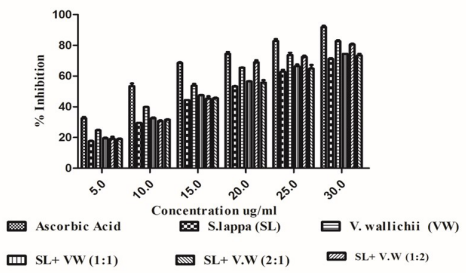

Figure 2: DPPH scavenging activity

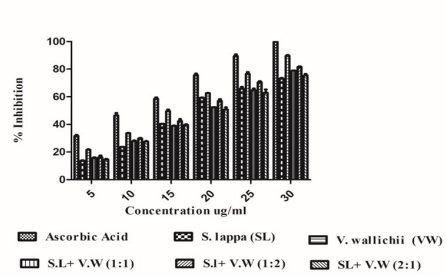

Figure 3: Hydroxyl radical scavenging activity

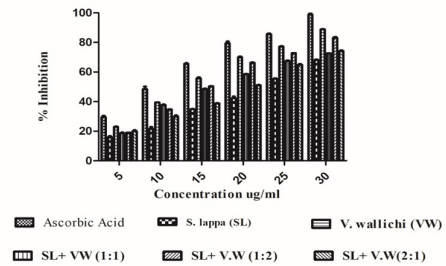

Figure 4: Oxide radical scavenging

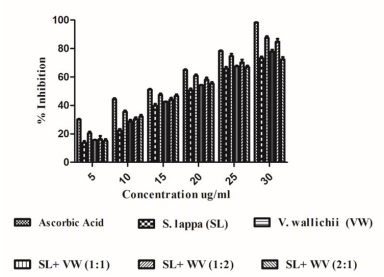

Figure 5: Superoxide radical scavenging activity

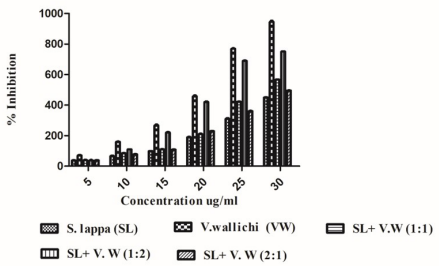

Figure 6: FRAP values (Fe2 + µmol/mL) of various extracts

Nitric Oxide Radical Scavenging[49]

Highly reactive, unstable species of nitric oxide combines with O_2 to form the stable compounds nitrate and nitrite. A decrease in nitrous acid in the presence of a scavenging agent estimated using Griess reagent at 546 nm can be the measure of antioxidant activity.[48] Ability of extracts to scavenge nitric oxide radicals, both singly and collectively, was assessed at various concentration levels, varying from 5.0 to 30.0 mg/mL, using ascorbic acid as the reference. All test samples' ability to scavenge nitric oxide radicals grew as concentration levels (Figure 4). *S. lappa* extract showed 68.12% scavenging, *V. wallichii* showed 88.88%, 1:1 ratio of both extracts showed 72.63%, 1:2 ratio of both extracts showed 83.23%, and 2:1 ratio of both extracts showed 74.17% at 30.0 mg/mL. In contrast, ascorbic acid at equal concentration exhibited 98.89% scavenging.

Superoxide Radical Scavenging Activity

Superoxide radical scavenging activity extracts of *S. lappa*, *V. wallichii* individually and in combination were determined at dissimilar concentration stages *viz*. 5.0 to 30.0 mg/mL taking ascorbic acid as standard shows 98.23 at 30.0 mg/mL (Table 7). extracts of *S. lappa* showed maximum inhibition of 73.12% at 30.0 mg/mL. *V. wallichii* extract showed maximum inhibition of 87.43% at 30.0 mg/mL. 1:1 ratio of both extracts showed 77.77%, 1:2 ratio of both extracts showed 84.82% and 2:1 ratio of both extracts showed 72.27% at 30.0 mg/mL.[50]

Ferric Reducing Antioxidant Power

The capability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the occurrence of 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ) is the principle of ferric-reducing antioxidant assay. The reaction is pH-dependent and takes place at an optimum pH of 3.6. The endpoint is Fe^{2+}-TPTZ complex with an intense blue color with an absorption max of 593 nm. Absorbance decreases with an increase in the antioxidant content.[51] Ferric reducing antioxidant power activity of extracts of *S. lappa* and *V. wallichi* individually and in combination was determined at dissimilar concentrations. 5.0 to 30.0 mg/mL taking ferrous sulfate as standard. The reducing capacity of all tested contents was uttered in terms of µmol Fe (II)/mL of the extract concentration compared with the ferrous sulfate standard curve (Figure 6). FRAP value of *V. wallichi* was more than all other samples. The FRAP values of test samples at various tested concentrations are presented in Figure 6.

IC_{50} Determination

IC_{50}, i.e., the sample concentration required to scavenge 50% of free radicals using free radical scavenging techniques, is known as the half maximal Inhibitory Concentration value. It is inversely proportional to the antioxidant property of the sample, meaning low IC_{50} values denote high antioxidant capacity.[52] Through interpolation from the linear regression analysis, the IC_{50} value was determined. (Figure 7). Outcomes showed that both extracts have very strong antioxidant activity, with *V. wallichii* having relatively higher antioxidant potential and close to that of ascorbic acid.

DISCUSSION

Chemicals known as antioxidants interact with and destroy free radicals (also known as free radical scavengers), preventing cellular damage caused by free radicals.[53] The body has some antioxidants called endogenous antioxidants to neutralize free radicals, but these are not sufficient, and external (exogenous) oxidants are required to be provided, primarily the diet or medicines.[54]

The *in-vitro* antioxidant perspective of *V. wallichii* extract individually and in combination was assay reducing DPPH. H_2O_2, nitric oxide, superoxide, and FRAP photometric assay. Both medications show high antioxidant activity equivalent to Vit C, which was utilized as the indication standard, according

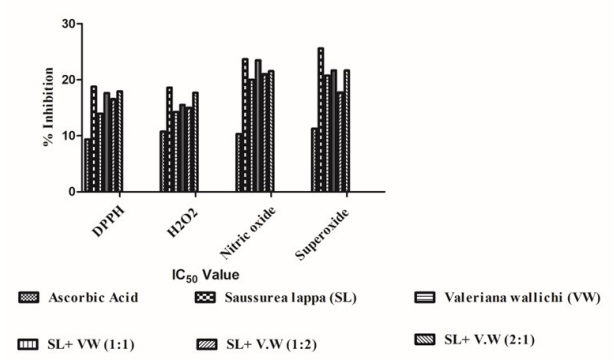

Figure 7: IC_{50} of extracts of *S. lappa*, *V. wallichii*, and Ascorbic acid

to the *in-vitro* antioxidant tests. By transferring hydrogen or electrons atoms,[55] antioxidant compounds neutralize the free radical nature of and create a colored moiety that can be measured colorimetrically using a spectrophotometer. The level of color or discoloration reveals the antioxidant's or extract's overall scavenging power in terms of hydrogen-donating ability.[56]

Extracts of *S. lappa* and *V. wallichii* showed potential antioxidant activity alone and in combination. But the activity was moderately addictive and not can be called synergistic, which is contrary to some observations of in vitro synergistic results for different plant extracts.[57-59] This might be due to the add-on effect of the antioxidant components of extracts. The plants may show synergistic effects *in-vivo* due to multicomponent multifaceted effects but not in *in-vitro*, where a chemical reaction is only a measure of antioxidant activity.

CONCLUSIONS

Many diseases are caused or aggravated due to the action of free radicles. The treatment by strong and potent antioxidants can be very useful in the management of such diseases. *S. lappa* and *V. wallichii* are traditionally used for many disorders and are reported to have potent antioxidant action. Outcomes showed very good antioxidant activity by both drugs evaluated using different methods. *V. wallichii* shows superior antioxidant activity than *S. lappa* when tested alone. When tested in combination, the extracts show an antioxidant effect equivalent to an additive effect and not a synergistic increment. This may be due to the involvement of chemical detection of antioxidant potential.

CONFLICTS OF INTEREST

There are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Both authors were involved in conceptualization and study design. Mrs. M. S. Potbhare did experimentation and manuscript preparation. Supervision, reviewed, and editing by Dr. Rakesh Barik. After reading, the final paper has been approved for publication by both authors.

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