Section A-Research paper

Assessment of Free Radical Scavenging Activity of *Acorus Calamus* Linn. and Alpha-Asarone Using in Vitro Models

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Abstract: The present study aimed to evaluate the free radical scavenging activity of *Acorus calamus* Linn. and its major bioactive compound, alpha-asarone, using in vitro models. The plant extract and alpha-asarone were subjected to various antioxidant assays such as DPPH, H_2O_2, total phenolic content, and total flavonoid content assay to determine their scavenging activity against free radicals. The results of the study indicated that both A. calamus extract and alpha-asarone showed significant antioxidant activity in a concentration-dependent manner. The DPPH assay showed IC_{50} values of 48.89 ± 0.31 µg/mL and 55.91 ± 0.28 µg/mL for A. calamus extract and alpha-asarone, respectively. The H_2O_2 assay showed IC_{50} values of 46.29 ± 0.31 µg/mL and 56.02 ± 0.33 µg/mL for A. calamus extract and alpha-asarone, respectively.

In conclusion, the study provides scientific evidence for the potential use of A. calamus extract and alpha-asarone as natural antioxidants in the prevention and treatment of oxidative stress-related diseases. Further in vivo studies are required to validate these findings and assess their therapeutic potential.

Keywords: Acorus *calamus* L., Alpha-asarone, Antioxidant

Abbreviations: AC: *Acorus calamus* Linn.; AA: Alpha-asarone, DPPH: 1,1-diphenyl-2-picrylhydrazyl; H_2O_2: Hydrogen peroxide; TFC: Total flavonoid content.

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Introduction: Antioxidants refer to substances that help shield the body from oxidative stress resulting from free radicals, which can inflict harm to cells and tissues. Free radicals are extremely reactive molecules capable of harming tissues and cells (Balmus *et al*., 2016). Oxidative stress has been linked to numerous chronic diseases, including diabetes, cancer, and cardiovascular diseases. The human body produces its own antioxidants, but the levels may not be sufficient to counteract the damage caused by free radicals (Mau *et al*., 2002; Adebayo *et al*., 2012).

Consequently, there has been an escalating interest in employing natural antioxidants, particularly those derived from plant sources. *Acorus calamus* Linn., commonly known as sweet flag, is a perennial herb that is widely distributed throughout the world, including Asia, Europe, and North America (Oli *et al*., 2021). AC has been used in traditional medicine for various ailments, such as fever, cough, and gastrointestinal disorders (Yadav *et al*., 2019). The plant contains several bioactive compounds, including AA, which has been found to exhibit significant antioxidant activity. AA is a phenylpropene-type compound that has been reported to possess several paralogical properties, including antioxidant, antimicrobial, and anti-inflammatory activities (Sarjan *et al*., 2017).

In this study, we aim to assess the in vitro antioxidant activity of AC and its active constituent AA using various assays, including the DPPH assay, H_2O_2 assay, total phenolic content, and total flavonoid content. The DPPH assay is a widely used method to assess a compound's potential for antioxidant activity. Which relies on the ability of antioxidants to counteract the stable DPPH radical. The H_2O_2 assay measures the compounds' ability to inhibit the generation of hydrogen peroxide, which is a reactive oxygen species. The assays for total flavonoid content and total phenolic content are employed to assess the phenolic and flavonoid constituents of the extracts, respectively, which are important contributors to the antioxidant activity of plant extracts.

The assess of the in vitro antioxidant activity of AC and its active constituent AA has several potential benefits. Firstly, it can provide valuable insights into the potential use of AC and AA as natural sources of antioxidants, which can help to lowering the risk of oxidative stress-related

diseases. Secondly, it can contribute to the development of natural antioxidant-based therapies for the prevention and management of chronic diseases. Thirdly, it can pave the way for further research on the bioactive compounds present in AC, which can potentially lead to the discovery of new therapeutic agents.

In conclusion, the evaluation of the in vitro antioxidant activity of AC and its active constituent AA can serve as a basis for further research on the therapeutic potential of this plant and its bioactive compounds. The findings of this investigation can yield significant insights for designing natural antioxidant-based treatments aimed at mitigating and managing chronic illnesses.

Procedure and Experimentation:

Chemicals and Reagents: Alpha-asarone, Catechin, gallic acid, ascorbic acid, aluminum chloride, sodium phosphate, sodium nitrite, hydrogen peroxide, 1,1-diphenyl-2-picrylhydrazyl, folin-ciocalteus`s phenol reagents used were of analytical grade procured from Sigma Aldrich.

Plant collection and Extraction: The dried AC rhizomes were purchased from a local traditional Indian medicine shop. The rhizomes were fragmented into small pieces, crushed, and soaked for three days in a container filled with a mixture of ethanol and water (1:1). Utilise a separating funnel to collect the active menstrual component-containing menstruum. The filtrated extract was concentrated using a rotary evaporator. Semi solid extract was lyophilized to obtained powder extract. Using formula, percentage yield of extract (14.4 w/w) was calculated. Powdered extract stored at cool temperature (Kalra et al., 2023).

Antioxidant Activity of the Plant Extracts

2,2-diphenyl-2-picryl-hydrazyl radical scavenging (DPPH) Assay:

Employing the DPPH assay, the extracts' potential to scavenge free radicals was evaluated. The decolorization of a methanol solution of DPPH was used to evaluate the plant extract capability to donate hydrogen atoms. It produces a purple coloration in methanol solution that fades to a yellow colour in the presence of an antioxidant (Sethi *et al*., 2020; Thaipong *et al*., 2006). Plant extract stock solution was made by dissolving extract in methanol at a concentration of 1mg/ml. Diluted concentrations of 100, 200, 300, 400, and 500 g/ml were produced from the stock solution. In the same way, a standard solution of ascorbic acid was made in the same concentration range. A 0.135

mM solution of DPPH was prepared in methanol. Then 1 milliliter of DPPH solution was combined with one milliliter of extracts with varying concentrations. The mixture was vigorously stirred and left in a dark place for 30 min. Following that, the absorbance of the mixture was determined at 517 nm by using a spectrophotometer. Blank was prepared without addition of sample. Absorbance of plant extract is compared with the standard solution (ascorbic acid) concentration.

Using following formula, inhibition percentage calculated

Inhibition Percentage (%) = [($A_c - A_s$) / (A_c)] x 100

Where A_c was absorbance of DPPH

A_s was absorbance of DPPH + sample (i.e., standard or extract)

Hydrogen peroxide (H_2O_2) radical scavenging activity:

The traditional approach for determining hydrogen peroxide (H_2O_2) scavenging activity of plant extract is to measure the disappearance of H_2O_2 at 230 nm. Several oxidase enzymes in the body produce hydrogen peroxide in vivo. Hydroxide peroxide scavenges by producing a hydroxyl radical as a reduction product. When scavenger is incubated with hydrogen peroxide in this approach, the decay and loss of hydrogen peroxides are detected spectrophotometrically at 230 nm. Decolorization of phosphate buffer solution of H_2O_2 was used to test the antioxidant activity of plant extract (Klein *et al*., 1981).

The stock solution of 1 mg/ml of plant extract was prepared in distilled water. Different concentrations of extract were prepared 100, 200, 300, 400, 500 µg/ml, by diluting the stock solution. Standard (Ascorbic acid) concentration was prepared in similar manner. In phosphate buffer solution (50 mM pH - 7.4), hydrogen peroxide (40 mM) is prepared.

1ml of extract and 0.6 ml of hydrogen peroxide was taken and mixed properly, The absorbance was measured at 230 nm after a 10-minute interval. The blank solution consisted of a phosphate buffer without the extract or standard solution. (Khan *et al*., 2013).

The percentage of hydrogen peroxide scavenging activity measured by using following formula

Inhibition Percentage (%) = [($A_{control} - A_{sample}$) / ($A_{control}$)] × 100

Where $A_{control}$ was absorbance of control

A_{sample} was absorbance of sample/standard.

Total phenolic content:

Plant secondary metabolites are phenolic compounds. These molecules have an aromatic ring with one or more hydroxyl groups and can be as simple as a phenolic molecule or as complex as a high-molecular-weight polymer. The number and position of the hydroxyl groups, as well as the type of aromatic ring modification, all influence the antioxidant activity of a phenolic molecule. Phenolic compounds have the ability to directly scavenge a variety of reactive species, such as peroxyl, hydroxyl and superoxide radicals, in order to perform their antioxidant activity. Some phenolic compounds can bind metals that promote oxidation, such as iron and copper, preventing the formation of free radicals.

The Folin-Ciocalteau reagent was used to measure the total phenolic content in the aqueous and ethanolic extracts of CO (Do *et al*., 2014; Youn *et al*., 2019). The construction of the calibration curve was performed by mixing 1 ml of Gallic acid solutions with different concentrations (20, 40, 60, 80, 100, and 120 mg/ml) with 5.0 ml of Folin Ciocalteu reagent that had been diluted tenfold and 4.0 ml of sodium carbonate solution with a concentration of 75 g/l. The mixture was incubated for 30 minutes and the absorbance was measured at a wavelength of 765 nm to plot the calibration curve, as shown in Figure 1.

To make the calibration curve, 1 mL of both the aqueous and ethanolic extracts with a concentration of 1 g/100 mL were separately combined with the same reagents. After one hour, the absorbance was measured in order to compute the total phenolic content in each extract individually using the following formula:

$C = C_1 + V/M$

C = total phenolic content in mg/g in gallic acid equivalent

C_1 = Concentration of gallic acid.

V = Volume of extract in ml

W-sample weight in gram.

Total Flavonoid content:

They specifically belong to a class of secondary plant metabolites with a polyphenolic composition that are found in various drinks, fruits, and vegetables. Flavonoids constitute a vast group of natural compounds. They possess numerous advantageous biochemical and antioxidant properties that have been linked to a number of diseases, including Alzheimer's disease, cancer, and atherosclerosis

(Chang *et al*., 2002; Park et al., 2008). Total flavonoid content is a measure of the quantity of flavonoids present in a sample. It can be determined using various chemical methods, including colorimetry, spectrophotometry, and high-performance liquid chromatography.

The total flavonoid contents of the AC and AA were estimated using the aluminum chloride colorimetric assay (Fattahi *et al*.,2014). The calibration curve (Figure 2) was plotted by mixing 0.5 ml aliquots of 20, 40, 60, and 80 mg/ml rutin solutions with 0.5 ml of 2% aluminum chloride solution. The absorbance was measured after one hour to calculate the total flavonoid content in each extract individually using the procedure.

$C = C_1 + V/M$

C = total flavonoid content in mg/g in rutin equivalent

C_1 = Concentration of rutin.

V = Volume of extract in ml

W-sample weight in gram.

Statistical analysis:

GraphPad Prism 9.5.1 software was used to conduct all analyses in triplicate, The outcomes were demonstrated as mean standard error of the mean (SEM) Analysis was done on the comparison of AC and AA at various concentrations.

Results:

Total Phenolic content:

The type of sample, solvent, and temperature employed for extraction all affect the polyphenol content. The phenolic content of AC was 42.75 mg GAE/gm of dry weight as shown in table 1. Standard curve of TPC is as shown in Figure 1.

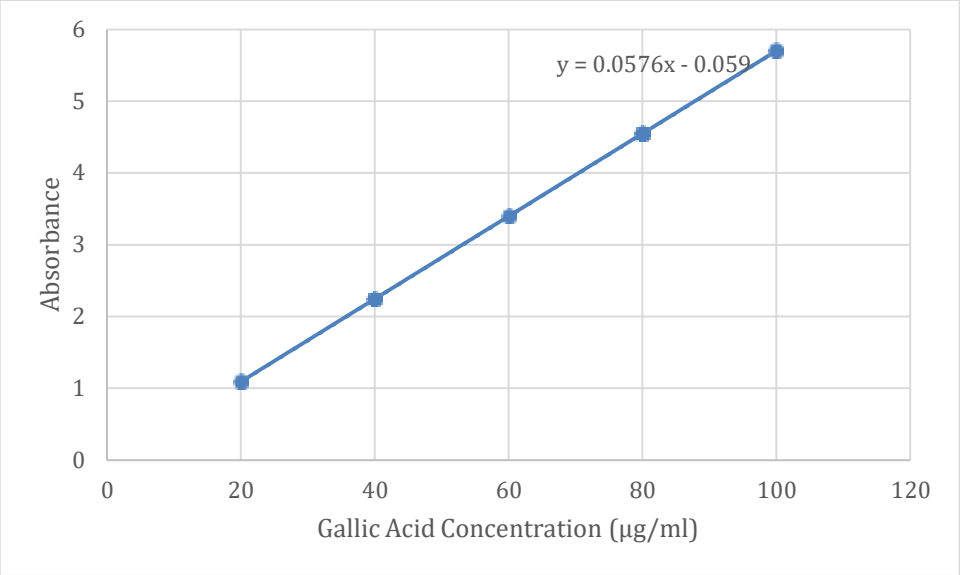

Figure 1: Standard curve for total phenolic content

Table 1: Total Phenolic Content of AC

Extract	Phenolic Content (mg GAE/g dry wt.)
Dried rhizomes of AC	42.75

Total Flavonoid Content: The quantity of polyphenols presents in the extract, as well as the extraction solvent and temperature, all affect the flavonoid content. The standard curve of flavonoid content is shown in figure 2. The total flavonoid content of AC was 22.75 as shown in table 2.

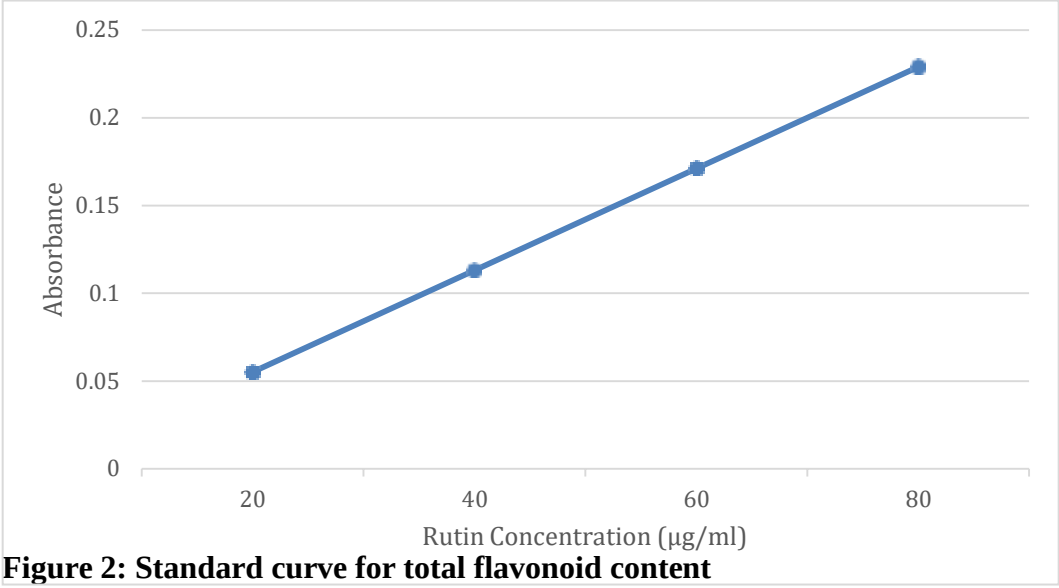

Figure 2: Standard curve for total flavonoid content

Table 2: Total flavonoid Content of AC

Extract	Flavonoid Content (mg RE/g dry wt.)
Dried rhizomes of AC	22.75

Hydrogen Peroxide Assay:

The AC and AA demonstrated remarkable scavenging activity in a dose dependent manner as shown in table. The standard (Ascorbic acid) exhibited significantly higher antioxidant activity ($P < 0.05$). At all the studied concentration, AA exhibited higher antioxidant activity as compared to AC as shown in Table 3 and figure 3.

Table 3: H_2O_2 scavenging activity of Ascorbic acid, AA and AC

Results H_2O_2			
Concentration	**% Inhibition**		
	Ascorbic acid	**AA**	**AC**
0	0	0	0
100	36.49±0.17	25.96±0.27	20.79±0.09
200	46.99±0.23	37.67±0.16	29.55±0.14
300	59.01±0.28	41.1±0.25	38.52±0.21
400	66.49±0.42	49.44±0.31	41.62±0.28
500	72.72±0.39	56.2±0.33	46.29±0.31

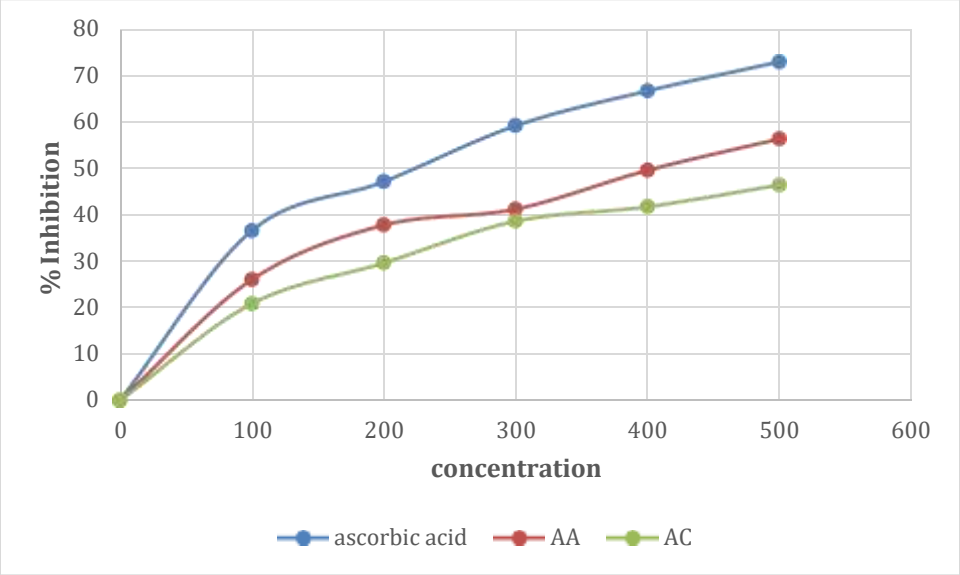

Figure 3: H_2O_2 scavenging activity of Ascorbic acid, AA and AC

DPPH Assay:

In a dose-dependent manner, AA and AC exhibited significant DPPH radical scavenging activity. As can be seen in table 4 and figure 4, the standard ascorbic acid had the highest scavenging activity, followed by AA, and then AC.

Table 4: DPPH scavenging activity of Ascorbic acid, AA and AC

Results DPPH			
Concentration	**% Inhibition**		
	A ascorbic	**AA**	**AC**
0	0	0	0
100	26.6±0.08	18.19±006	13.12±0.05
200	37.08±0.13	25.23±0.1	20.21±0.09
300	49.6±0.21	35.14±0.17	28.37±0.12
400	60.56±0.29	50.65±0.26	44.51±0.24
500	69.79±0.34	55.91±0.28	48.89±0.31

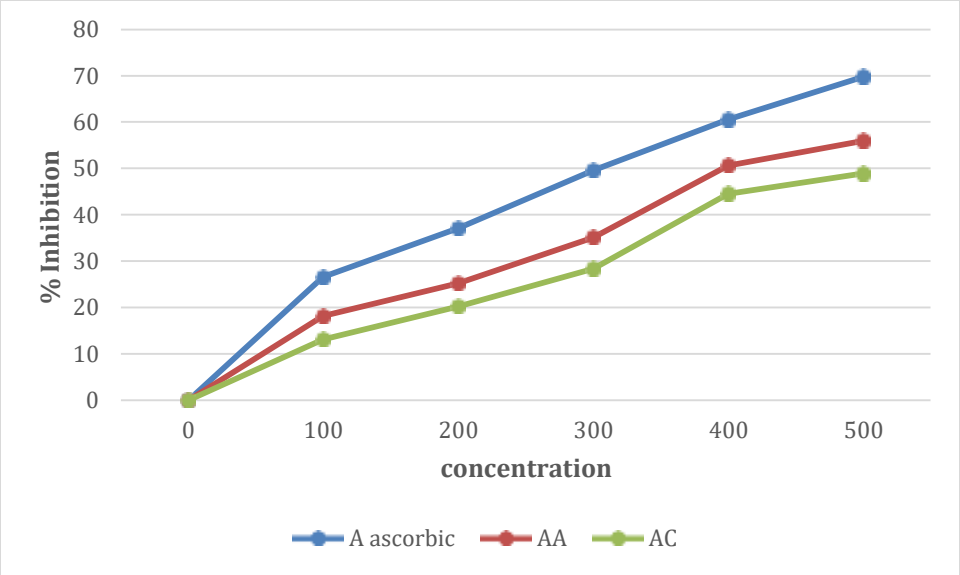

Figure 4: DPPH scavenging activity of Ascorbic acid, AA and AC

Discussion:

The findings from the study suggest that the extract of dried rhizomes of AC and its active constituent AA possess antioxidant properties. The DPPH assay and H2O2 assay are frequently

employed techniques to assess the antioxidant potential of plant extracts in scientific research, and the results of these assays indicate that both the extract and AA have antioxidant properties.

The total phenolic content and total flavonoid content are often used as indicators of antioxidant activity in plant extracts. The higher values of total phenolic and flavonoid content in plant extract suggest that it may have a greater potential to scavenge free radicals and prevent oxidative damage.

It is noteworthy that AA exhibited higher antioxidant activity than the extract in both the DPPH and H_2O_2 assays. This could be due to the fact that AA is a more concentrated form of the active constituents found in the extract, and thus may exhibit greater antioxidant activity.

The results of your study are consistent with previous reports that have demonstrated the antioxidant potential of AC and its constituents. The presence of phenolic compounds and flavonoids in AC is thought to account for its antioxidant activity, owing to the well-established strong antioxidant properties of these compounds.

Overall, your study provides evidence to support the use of AC and its active constituent AA as potential sources of natural antioxidants. However, further investigations are required to elucidate the mechanism of action of these compounds and appraise they're in-vivo antioxidant activity potential.

Conclusion:

The results of this study indicate that the extract of dried rhizomes of AC and its active constituent AA possess antioxidant properties. The DPPH and H_2O_2 assays showed that both the extract and AA have antioxidant activity, with AA exhibiting higher activity than the extract. These results indicate that AC and AA have the potential to serve as natural sources of antioxidants that could have various health benefits. However, furthermore research is necessary to elucidate the mechanism of action of these compounds and assess their potential in vivo antioxidant activity. The results of this study also highlight the importance of evaluating the

antioxidant potential of natural products, which may have potential applications in the development of new therapeutic agents.

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