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Antioxidant Activity and Effects of Kedayan (*Aristolochia* sp.) Stem Extract on HSC-3 Human Oral Cancer Cells

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ABSTRACT

Medicinal plants are a promising source of bioactive compounds with potential therapeutic applications, including antioxidant and anticancer-related activities. This study aimed to identify secondary metabolites in the stem extract of *Aristolochia* sp. and to evaluate its antioxidant activity and effects on the viability of HSC-3 human oral cancer cells. The stem extract was prepared by maceration with 95% ethanol and screened qualitatively for phytochemical constituents. Antioxidant activity was assessed using the DPPH assay, whereas cell viability was evaluated using the CCK-8 assay. The extract contained several secondary metabolites, including flavonoids, phenolic compounds, saponins, and tannins. In the DPPH assay, the extract showed an IC_{50} value of 138.34 ± 12.14 µg/mL, indicating moderate antioxidant activity. In the HSC-3 viability assay, the extract reduced cell viability in a concentration-dependent manner; however, the IC_{50} was not reached within the tested concentration range and was therefore reported as >50 µg/mL. The greatest reduction in HSC-3 cell viability was observed at 50 µg/mL, with a corresponding cell mortality rate of 39.90%, which differed significantly from the control groups ($p < 0.05$). In conclusion, the stem extract of *Aristolochia* sp. demonstrated moderate antioxidant activity and reduced HSC-3 cell viability within the tested concentration range. These findings provide preliminary evidence of bioactivity; however, further studies using a wider concentration range and additional mechanistic and safety evaluations are required before anticancer potential can be inferred.

Keywords:
Aristolochia sp.; Stem extract; Antioxidant activity; HSC-3 cells; Cell viability

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1. Introduction

Indonesia is a tropical archipelagic country located between two continents and is recognized as one of the most biodiverse countries in Asia [1]. Approximately 25% of the world's flowering plant species are found in Indonesia, with around 20,000 species

recorded and a substantial proportion considered endemic [2]. Kalimantan is one of the regions with particularly high plant diversity compared with other parts of Indonesia [3]. However, the potential of these genetic resources remains insufficiently explored. Only a limited number of plant species have been documented with respect to their medicinal applications and bioactive potential [4]. Medicinal plants are widely regarded as promising sources of natural compounds because they contain metabolites that may contribute to the prevention or treatment of various diseases, including cancer [5].

One plant with traditional medicinal relevance in North Kalimantan is *Aristolochia* sp., locally known as Kedayan. According to community reports, it has been used for wound healing, pain relief, and as an antidote [6]. Despite this traditional use, its biological activity has not been adequately characterized [7]. The stem and root are the plant parts most commonly used for medicinal purposes. Previous studies on the root of *Aristolochia* sp. have identified several secondary metabolites, including flavonoids, alkaloids, and tannins, which are known to possess antioxidant-related properties [8]. At the same time, many *Aristolochia* species are known to contain aristolochic acids, compounds associated with nephrotoxic and carcinogenic effects [9]. Therefore, any investigation of *Aristolochia* sp. should consider not only its potential bioactivity but also the need for caution regarding safety and toxicological risk [10].

Antioxidants play an important role in protecting cells against oxidative damage caused by free radicals. Several classes of secondary metabolites, including flavonoids and alkaloids, have been reported to support tissue repair and wound healing [11], whereas tannins may contribute to hemostatic activity through protein precipitation and vascular stabilization [12]. In addition to these antioxidant-related effects, it has been hypothesized that extracts containing antioxidant-active compounds may also show antiproliferative activity against cancer cells. However, this relationship is not universal and may vary depending on the extract composition, assay system, and cell type under investigation [13],[14]. For this reason, direct evaluation of both antioxidant activity and cancer cell response is necessary.

Although *Aristolochia* sp. has recognized ethnomedicinal use and preliminary evidence of antioxidant-related phytochemicals in its root, information on the stem extract remains limited, particularly regarding its activity against oral cancer cells. The HSC-3 cell line is a relevant in vitro model for studying oral squamous cell carcinoma and may therefore be useful for initial screening of plant-derived bioactivity. Accordingly, this study aimed to identify the secondary metabolites present in the stem extract of *Aristolochia* sp. and to evaluate its antioxidant activity and effects on the viability of HSC-3 human oral cancer cells. Through this approach, the study was intended to provide preliminary evidence regarding the biological activity of *Aristolochia* sp. stem extract while also supporting future investigations into its potential and safety profile.

2. Methods

Extraction of *Aristolochia* sp. Stems

The stems of *Aristolochia* sp. were collected from a forest area in Malinau, East Kalimantan, Indonesia. The plant material was identified by the National Research and Innovation Agency of Indonesia (BRIN) under registration number B-537/V/DI.0507/11/2021. Fresh stems (800 g) were oven-dried at 40 °C until a constant weight was obtained, yielding 200 g of dried material. The dried stems were then powdered and macerated with 2 L of 95% ethanol until the solvent became colorless. The extract was filtered and concentrated using a rotary evaporator to obtain the crude

extract [15]. The extraction process was carried out at the Center for Standard Testing of Spices, Medicinal Plants and Aromatic Plants (BPSI-TROA). For biological assays, the crude extract was prepared as serial concentrations of 10, 20, 30, 40, and 50 µg/mL [7].

Phytochemical Screening of *Aristolochia* sp. Stem Extract

Phenolic and Flavonoid Tests

Phenolic compounds were tested by dissolving the extract in ethanol followed by the addition of four drops of 5% iron(III) chloride solution. The appearance of a blue coloration indicated the presence of phenolic compounds. For flavonoid testing, the extract was dissolved in ethanol and treated with 0.1 mg magnesium powder, five drops of 37% hydrochloric acid, four drops of 2 N sulfuric acid, and four drops of 10% sodium hydroxide. The formation of an orange color indicated the presence of flavonoids [16],[17].

Steroid and Triterpenoid Tests

For steroid and triterpenoid testing, the extract was mixed with ether, filtered, and evaporated. The residue was then treated with Liebermann–Burchard reagent. A blue-green color indicated the presence of steroid compounds, whereas a purple color indicated the presence of triterpenoid compounds [18].

Saponin and Tannin Tests

To test for saponins, the extract was mixed with distilled water, heated for 15 min, and shaken for 30 s. The formation of stable foam that persisted after the addition of hydrochloric acid indicated a positive saponin reaction [17]. For tannin testing, the extract was dissolved in ethanol and treated with four drops of 1% iron(III) chloride solution. A blue-green coloration confirmed the presence of tannins [19].

Antioxidant Activity Test of *Aristolochia* sp. Extract

Antioxidant activity was evaluated using the DPPH assay in triplicate (n = 3). The extract was tested at concentrations of 50, 100, 150, 200, and 250 µg/mL. Sample solutions were prepared and mixed with DPPH reagent (Sigma-Aldrich), then incubated for 30 min in the dark at room temperature. Absorbance was measured at 517 nm using a Shimadzu UV-1800 UV–Vis spectrophotometer. Methanol containing DPPH without extract was used as the control solution, and methanol without DPPH was used as the reagent blank. The percentage of radical scavenging activity was calculated based on the difference in absorbance between the control and sample solutions [20]. If ascorbic acid was used as a reference antioxidant in the experiment, its concentration range and IC_{50} value should also be reported in the Results section for consistency.

Cytotoxicity of *Aristolochia* sp. Extract on HSC-3 Cells

The effect of *Aristolochia* sp. stem extract on the viability of HSC-3 human oral cancer cells was evaluated using the Cell Counting Kit-8 (CCK-8) assay. The extract was tested at concentrations of 10, 20, 30, 40, and 50 µg/mL. HSC-3 cells were obtained from the biorepository of YARSI University and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antimycotic–antibiotic solution. The cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. Three control groups were included, namely untreated HSC-3 cells as the negative control, a solvent control containing the same vehicle used for extract preparation, and a cytotoxic control containing 10% DMSO [21]. After seeding into 96-well plates, the culture medium was discarded and the wells were rinsed once with phosphate-buffered saline (PBS). The cells were then treated with the extract at each concentration and incubated for 24 h. Cell viability was assessed by adding CCK-8 reagent (Sigma-Aldrich), followed by an additional 1 h incubation, and absorbance was measured at 450 nm using a microplate reader [20].

The cytotoxic effect of the extract was expressed as the percentage reduction in cell viability relative to the untreated control. In this study, the percentage of cell death was calculated using the following equation:

$$\text{Cell death (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where A_{control} represents the absorbance of untreated control cells and A_{sample} represents the absorbance of extract-treated cells. A higher percentage value indicates greater cytotoxic activity of the extract against HSC-3 cells. Because the tested concentration range did not reach 50% inhibition, the cytotoxic response was interpreted descriptively within the tested range, and the IC_{50} was therefore reported only as >50 µg/mL rather than as a definitive calculated value.

Statistical Data Analysis

Data were analyzed using the Shapiro–Wilk test for normality, followed by one-way analysis of variance (ANOVA) and Tukey's post hoc test. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS). A p-value < 0.05 was considered statistically significant.

3. Results and Discussion

Phytochemical Profile of *Aristolochia* sp. Stem Extract

Qualitative phytochemical screening of the ethanol stem extract of *Aristolochia* sp. revealed the presence of several secondary metabolites, as presented in **Table 1**. The extract tested positive for phenolic compounds, flavonoids, triterpenoids, saponins, and tannins, whereas steroid compounds were not detected. These findings indicate that the stem extract contains multiple classes of phytochemicals that are commonly associated with antioxidant-related and other biological activities.

The detection of phenolic compounds and flavonoids is particularly important because these metabolites are widely recognized as major contributors to the antioxidant capacity of medicinal plants. In addition, tannins may contribute to biological activity through their protein-binding and hemostatic properties, whereas saponins and triterpenoids are also frequently reported as bioactive constituents in medicinal plant extracts [11],[12],[27]. The absence of steroid compounds in the present extract suggests that the phytochemical composition of the stem may differ from that of other plant parts or may depend on the extraction conditions used in this study.

Table 1. Qualitative Phytochemical Test Results of *Aristolochia* sp. Stem Extract

Secondary metabolite	Reagent	Observation	Interpretation
Phenolic compounds	5% $FeCl_3$	Green color	Positive (+)
Flavonoids	Concentrated HCl + Mg; 2 N H_2SO_4; 10% NaOH	Yellow to orange color	Positive (+)
Steroids	Liebermann–Burchard reagent	No color change	Negative (−)
Triterpenoids	Liebermann–Burchard reagent	Brown color	Positive (+)
Saponins	HCl + H_2O	Stable foam/bubbles	Positive (+)
Tannins	1% $FeCl_3$	Green color	Positive (+)

The phytochemical profile observed in the present study is broadly consistent with previous reports on the root of *Aristolochia* sp., which identified flavonoids, tannins, and

other antioxidant-related metabolites in the root extract [6],[8]. Although the present study focused on the stem rather than the root, the detection of overlapping classes of secondary metabolites suggests that different parts of *Aristolochia* sp. may share a number of potentially bioactive constituents. Nevertheless, the relative composition and abundance of these compounds may vary depending on the plant part, extraction solvent, and preparation method.

Taken together, these findings provide an initial phytochemical basis for the subsequent evaluation of antioxidant activity and HSC-3 cell viability. However, because the present analysis was qualitative, further analytical studies are still needed to isolate, identify, and quantify the major constituents responsible for the observed biological activity. This is particularly important for *Aristolochia* species, given that this genus may also contain compounds of toxicological concern and therefore requires careful phytochemical characterization in future studies [9],[10].

Antioxidant Activity of *Aristolochia* sp. Stem Extract

The antioxidant activity of the ethanol stem extract of *Aristolochia* sp. was evaluated using the DPPH radical scavenging assay, and the results are presented in **Table 2**. The assay was performed in triplicate, and the results are expressed as mean ± SD. The extract showed a concentration-dependent increase in radical scavenging activity across the tested concentration range of 50-250 µg/mL. The mean percentage inhibition increased from 25.37 ± 3.73% at 50 µg/mL to 74.52 ± 2.13% at 250 µg/mL, indicating that higher extract concentrations were associated with greater DPPH radical scavenging capacity.

Table 2. DPPH Radical Scavenging Activity of *Aristolochia* sp. Stem Extract

Extract concentration (µg/mL)	Inhibition (%) Replicate 1	Inhibition (%) Replicate 2	Inhibition (%) Replicate 3	Mean ± SD inhibition (%)	IC_{50} (µg/mL)
50	21.06	27.66	27.38	25.37 ± 3.73	138.34 ± 12.14
100	40.91	45.83	45.95	44.23 ± 2.87	-
150	51.62	57.64	56.99	55.42 ± 3.30	-
200	61.49	65.28	64.98	63.92 ± 2.11	-
250	72.08	76.04	75.44	74.52 ± 2.13	-

Note: *Antioxidant activity was measured in triplicate (n = 3) using the DPPH assay. Results are presented as mean ± SD. The IC_{50} value was derived from linear regression of the three replicate measurements.*

Based on linear regression of the three replicate measurements, the IC_{50} value of the *Aristolochia* sp. stem extract was calculated as 138.34 ± 12.14 µg/mL. This value reflects the concentration required to inhibit 50% of DPPH radicals and indicates a measurable antioxidant effect of the extract. The increasing inhibition percentages observed with rising extract concentrations also support the expected dose-dependent trend of antioxidant activity in the DPPH assay.

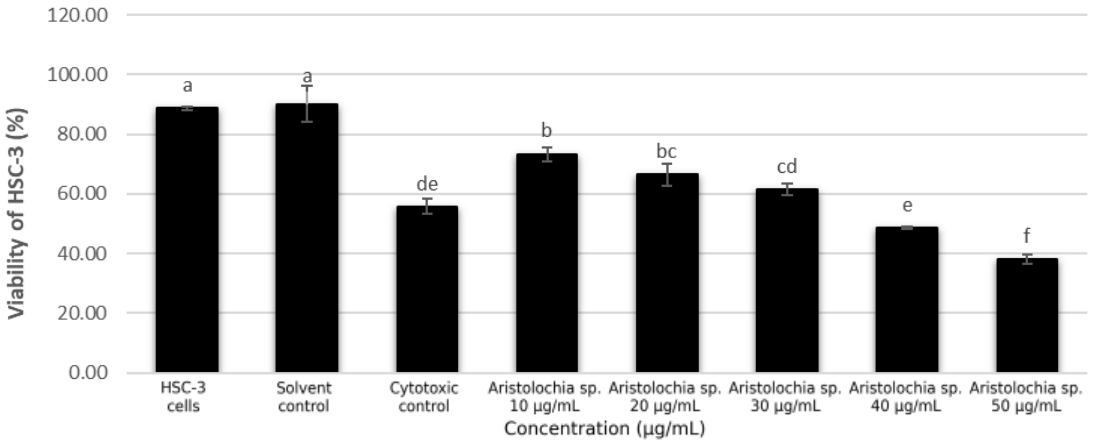
According to the antioxidant classification used in this study, IC_{50} values of 100-150 µg/mL are categorized as moderate. Therefore, the stem extract of *Aristolochia* sp. can be classified as having moderate antioxidant activity rather than strong or potent antioxidant capacity. This interpretation is consistent with the findings of Novermawati et al., who reported an IC_{50} value of 139.11 µg/mL for the ethyl acetate fraction of *Aristolochia* sp. root and similarly classified it as a moderate antioxidant [22], [23]. The close similarity between the present IC_{50} value and that reported previously suggests

that different parts of *Aristolochia* sp. may retain comparable antioxidant-related phytochemical activity, although the extract composition may differ depending on the plant part and extraction solvent.

The antioxidant activity observed in the present study is likely related to the presence of phenolic compounds and flavonoids identified in the phytochemical screening, as these metabolites are widely recognized for their capacity to donate electrons or hydrogen atoms to neutralize free radicals. Although antioxidant-rich plant extracts are often discussed in relation to broader biological benefits, the present findings should be interpreted specifically as evidence of moderate radical scavenging activity in the DPPH system. Additional studies are still required to determine how this antioxidant activity relates to other biological effects of the extract in more complex cellular systems [14].

Effects of *Aristolochia* sp. Stem Extract on HSC-3 Cell Viability

The effect of *Aristolochia* sp. stem extract on HSC-3 cell viability is presented in **Figure 1**. Within the tested concentration range of 10-50 µg/mL, the extract reduced HSC-3 cell viability in a concentration-dependent manner. The highest viability was observed in the negative control group (88.60%), followed by the solvent control group (90.10%), whereas progressively lower viability was observed in extract-treated groups as the concentration increased. The respective viability values for the extract-treated groups were 73.36%, 66.44%, 61.57%, 48.66%, and 38.04% at concentrations of 10, 20, 30, 40, and 50 µg/mL, respectively. The lowest viability among the tested extract concentrations was therefore observed at 50 µg/mL.



a-f in the column indicates a significant difference ($p < 0.05$). The lowercase letter 'a' shows that the number is higher compared to group 'b', while 'ab' shows that the difference is not significant ($p>0.05$).

Figure 1. Effects of *Aristolochia* sp. stem extract on HSC-3 cell viability after 24 h of treatment

Because 50% inhibition was not reached within the tested concentration range, the cytotoxic response of the extract could not be used to determine a definitive IC_{50} value. Accordingly, the result is more appropriately reported as $IC_{50} > 50$ µg/mL rather than interpreted as a calculated cytotoxic IC_{50}. Under these conditions, the findings indicate that the extract reduced HSC-3 cell viability within the tested range, but additional testing at higher concentrations would be required to determine the actual IC_{50} value. This interpretation is consistent with the reviewer's earlier concern that an IC_{50} should not be claimed outside the tested dose range.

Statistical analysis using one-way ANOVA followed by Tukey's post hoc test, as described in the Methods section, showed significant differences among groups, as indicated by the letter annotations in **Figure 1**. The negative control group differed significantly from the cytotoxic control and from the extract-treated groups at higher concentrations, while some adjacent treatment concentrations did not differ significantly from one another. In particular, no significant difference was observed among the 10, 20, and 30 µg/mL groups, and no significant difference was observed between the 40 and 50 µg/mL groups. However, the lower-concentration groups differed significantly from the higher-concentration groups, indicating that increasing extract concentration was associated with reduced HSC-3 cell viability. If available, the exact p-values from the ANOVA and Tukey analyses should be inserted here to fully match the reviewer's request and strengthen statistical reporting.

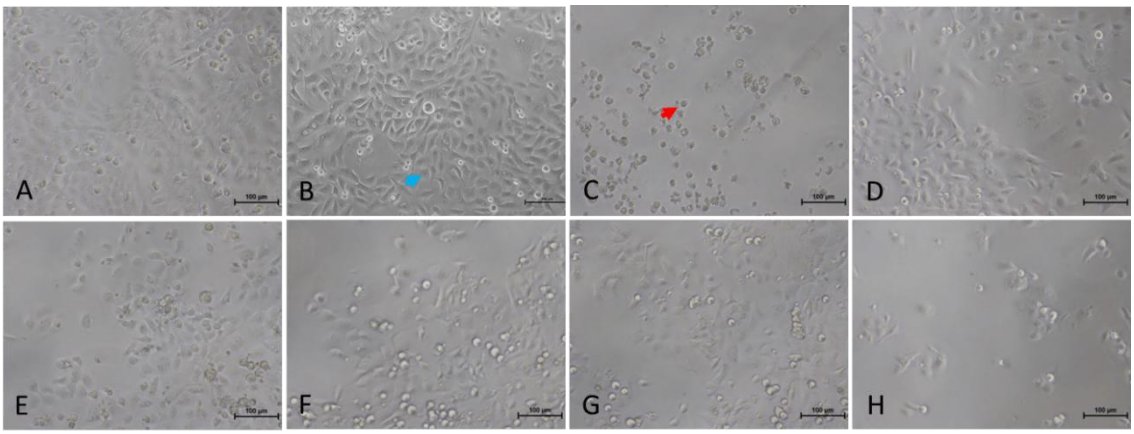


Figure 2. Effect of *Aristolochia* sp. extract on the Viability of HSC-3 Cells.Blue arrows indicate live HSC-3 cells with clearly visible nuclei, while red arrows indicate dead HSC-3 cells showing cell shrinkage. Morphological observations of HSC-3 cells are shown for each group: A. Negative control; B. Solvent control; C. Positive control; D. Extract at 10 µg/mL; E. Extract at 20 µg/mL; F. Extract at 30 µg/mL; G. Extract at 40 µg/mL; H. Extract at 50 µg/mL. Observations were made at 100x magnification.

Microscopic observations further supported the quantitative viability findings. As shown in **Figure 2**, viable HSC-3 cells with clearly visible nuclei were still present in all groups, but extract-treated cells increasingly showed morphological features associated with cell damage or death, including cell shrinkage. These morphological changes became more apparent as extract concentration increased, supporting the interpretation that the extract exerted a concentration-related effect on HSC-3 cell viability within the tested range.

The reduction in HSC-3 viability observed in this study may be related to the presence of secondary metabolites detected in the phytochemical screening, including flavonoids, tannins, saponins, and triterpenoids. Similar classes of compounds have been reported to contribute to antiproliferative or cytotoxic effects in cancer-related models [23]–[26]. Nevertheless, because the present study did not investigate the underlying mechanism of action, these findings should be interpreted as preliminary evidence of reduced HSC-3 viability rather than as definitive proof of anticancer activity. Further studies using a wider concentration range and mechanistic assays are needed to clarify the biological significance of the observed effect.

Integrated Interpretation of Antioxidant Activity and HSC-3 Viability Response

Taken together, the findings of the present study indicate that the stem extract of *Aristolochia* sp. possesses measurable bioactivity in two related but distinct experimental systems. In the DPPH assay, the extract showed moderate radical-scavenging activity, whereas in the HSC-3 viability assay, it reduced cell viability in a concentration-dependent manner within the tested range. These results suggest that the extract contains bioactive constituents capable of interacting with both chemical free-radical systems and cellular responses. However, these two observations should not be interpreted as direct proof of a causal relationship between antioxidant capacity and antiproliferative activity.

The phytochemical screening results support the possibility that the observed bioactivity is associated with the presence of phenolic compounds, flavonoids, tannins, saponins, and triterpenoids. Several of these classes of secondary metabolites have been reported to contribute to antioxidant effects and, in some cases, to reduced proliferation or increased death of cancer-related cell lines [13],[14],[23]–[26]. Nevertheless, the biological effect of a crude plant extract depends on the combined action of multiple constituents, their concentrations, and their interactions within a given assay system. Therefore, although the present phytochemical profile provides a plausible basis for both antioxidant activity and reduced HSC-3 viability, the responsible compounds and their mechanisms of action cannot yet be identified from the current data alone.

The current findings also indicate that moderate antioxidant activity does not automatically translate into confirmed anticancer activity. In the present study, the extract reduced HSC-3 cell viability, but a definitive cytotoxic IC_{50} could not be determined because 50% inhibition was not reached within the tested concentration range. Accordingly, the viability data should be interpreted as preliminary evidence of reduced HSC-3 cell viability rather than as conclusive evidence of strong cytotoxic or anticancer potency. This distinction is important, because antioxidant-rich extracts may show variable cellular effects depending on cell type, extract composition, concentration range, and experimental conditions [13],[14].

The microscopic observations further strengthen this interpretation by showing morphological changes consistent with reduced cellular viability at higher extract concentrations. When considered together with the concentration-dependent decline in viability, these findings suggest that the extract exerts a biologically relevant effect on HSC-3 cells within the tested range. Even so, the present study did not evaluate apoptosis, necrosis, cell-cycle arrest, oxidative stress signaling, or other molecular mechanisms. Therefore, the results should be regarded as an initial bioactivity profile rather than a mechanistic demonstration of anticancer action.

Overall, the combined antioxidant and HSC-3 viability findings suggest that the stem extract of *Aristolochia* sp. contains biologically active constituents worthy of further investigation. At the same time, the interpretation must remain cautious. The present study supports the existence of moderate antioxidant activity and a concentration-dependent reduction in HSC-3 cell viability, but additional studies using wider concentration ranges, compound isolation, and mechanistic assays are required before stronger conclusions can be drawn regarding therapeutic relevance or anticancer potential.

Safety Implications and Study Limitations

The findings of this study should be interpreted with caution from both efficacy and safety perspectives. Although the stem extract of *Aristolochia* sp. demonstrated moderate antioxidant activity and reduced HSC-3 cell viability within the tested concentration range, these results do not yet provide sufficient evidence to support a definitive anticancer claim. This point is particularly important because the cytotoxic IC_{50} was not reached at concentrations up to 50 µg/mL, indicating that the current assay range was insufficient to determine the actual inhibitory concentration required for 50% reduction in cell viability. Accordingly, the present data should be regarded as preliminary evidence of bioactivity rather than conclusive proof of cytotoxic potency against HSC-3 cells. In line with this limitation, further studies using a wider concentration range are required to establish a valid IC_{50} value and to better define the dose-response profile of the extract.

Safety considerations are also particularly relevant because many species within the genus *Aristolochia* are known to contain aristolochic acids, which have been associated with nephrotoxicity, genotoxicity, and carcinogenic risk [9], [10]. Therefore, the observation of biological activity in the present study should not be interpreted as evidence of therapeutic safety. Instead, it underscores the need for careful toxicological characterization of the extract, including identification of hazardous constituents and evaluation of their concentration in the stem extract. In this context, any future development of *Aristolochia* sp. as a bioactive natural product should proceed only after rigorous safety assessment.

Several methodological limitations should also be acknowledged. First, the study was limited to in vitro assays and therefore cannot predict in vivo efficacy or safety. Second, the phytochemical analysis was qualitative and did not identify or quantify the specific compounds responsible for the observed biological effects. Third, the study did not include mechanistic assays to determine whether the reduction in HSC-3 viability was associated with apoptosis, necrosis, cell-cycle arrest, oxidative stress modulation, or other pathways. Finally, the use of a restricted concentration range in the cytotoxicity assay prevented definitive classification of cytotoxic potency. Taken together, these limitations indicate that the current findings provide only an initial biological profile of *Aristolochia* sp. stem extract. Further studies should include expanded cytotoxicity testing, compound isolation and characterization, mechanistic evaluation, and comprehensive safety assessment before any stronger conclusions regarding therapeutic relevance can be drawn.

4. Conclusion

The ethanol stem extract of *Aristolochia* sp. demonstrated moderate antioxidant activity in the DPPH assay, with an IC_{50} value of 138.34 µg/mL. In the HSC-3 cell viability assay, the extract reduced cell viability in a concentration-dependent manner within the tested concentration range. However, because the IC_{50} was not reached at concentrations up to 50 µg/mL, the cytotoxic response could only be reported as IC_{50} > 50 µg/mL. These findings provide preliminary evidence that the stem extract of *Aristolochia* sp. contains bioactive constituents with measurable antioxidant activity and effects on HSC-3 cell viability. Nevertheless, further studies using a wider concentration range, detailed phytochemical characterization, mechanistic assays, and comprehensive safety evaluation are required before stronger conclusions can be made regarding its anticancer relevance or therapeutic potential.

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Conflicts of Interest:

The authors declare that there is no conflict of interest regarding the conduct of this research or the publication of this article.

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