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In Vitro Evaluation of Antioxidant and Anticancer Activity of *Alpinia nigra* and *Hedychium coronarium* Rhizomes of Manipur

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ABSTRACT: *Alpinia nigra* and *Hedychium coronarium* are two plants native to the biodiverse region of Manipur, known for their traditional therapeutic uses. The rhizomes of these plants are commonly used in local cuisine, particularly in the dish "eromba". With an emphasis on their total phenolic content (TPC), total flavonoid content (TFC), ability to scavenge free radicals, and cytotoxic effects on cancer cell lines, this study sought to assess the antioxidant and anticancer properties of these rhizomes. The anticancer activity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on A549 (lung adenocarcinoma) and HeLa (cervical adenocarcinoma) cell lines, while the antioxidant potential was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. The findings showed that the rhizomes of *Alpinia nigra* and *Hedychium coronarium* both demonstrated strong antioxidant activity (103.74 ± 2.54 and 373.15 ± 5.19 µg/ml), with high TPC (132.75 ± 0.37 (mg GAE/100g) and 83.57 ± 0.72 (mgQE/100g)) and TFC values (67.28 ± 1.58 (mg GAE/100g) and 46.26 ± 2.02 (mgQE/100g)) corresponding to their capacity to reduce ferric ions and scavenge free radicals. Additionally, the extracts showed dose-dependent cytotoxicity against HeLa and A549 cells, suggesting that they may have anticancer properties. These results demonstrate the plants' potential as natural sources of antioxidants and anticancer chemicals while also offering scientific support for their traditional use in Manipur. The study emphasizes how crucial it is to investigate Manipur's abundant biodiversity in order to create new medicinal compounds that will fight cancer and disorders linked to oxidative stress.

Keywords: *Alpinia nigra, Hedychium coronarium,* TPC, TFC, antioxidant activity, anticancer activity.

INTRODUCTION

The northeastern region of India, particularly Manipur, is a biodiversity hotspot, known for its abundant flora and ancient healing methods. Because of their widespread usage in traditional medicine and possible medicinal benefits, *Alpinia nigra* and *Hedychium coronarium* stand out among the many plant species found in this area. The Zingiberaceae family, which includes both plants, is well-known for its fragrant and therapeutic qualities. The indigenous populations of Manipur have long utilized the rhizomes of these plants to cure a variety of illnesses, such as microbial infections, digestive issues, and inflammation (Devi *et al*., 2014). The bioactive substances found in these rhizomes have drawn more attention in recent years, especially because of their potential to produce new therapeutic agents due to their anticancer and antioxidant qualities.

Because of its distinct climate and geography, Manipur is home to a vast array of medicinal plants that have been utilized in traditional treatment methods for generations. The medicinal properties of *Alpinia nigra* (called locally as "Pullei") and *Hedychium coronarium* (also known as "Loklei") have long been acknowledged by the state's traditional knowledge systems, especially those of the Meitei, Naga, and Kuki groups. These two are particularly used as vegetables in the preparation of the local cuisine called “eromba” which is a traditional, flavorful, and spicy dish made by boiling vegetables and spices, and then mashing them together with fermented fish. These plants are prized for their cultural relevance in daily life and rituals, in addition to their therapeutic qualities (Devi *et al*., 2014). But even with their extensive use, there is still no scientific proof of their pharmacological qualities, especially when considering contemporary therapeutic uses like the treatment of cancer and the control of oxidative stress.

Numerous chronic diseases, including as cancer, cardiovascular disorders, and neurological conditions, are linked to oxidative stress, which is brought on by an imbalance between the body's antioxidant defenses and the formation of reactive oxygen species (ROS) (Halliwell, 2012). Antioxidants are essential for reducing oxidative stress and averting cellular damage because they neutralize ROS. Because of their capacity to scavenge free radicals and alter pathways linked to

oxidative stress, natural products—especially those derived from plants—have drawn a lot of interest as possible sources of antioxidants (Lobo *et al*., 2010). Particularly well-known for their strong antioxidant properties, phenolic chemicals and flavonoids have been thoroughly investigated for their potential health advantages (Pandey & Rizvi 2009). Thus, assessing the rhizomes of *Alpinia nigra* and *Hedychium coronarium* for total phenolic content (TPC) and total flavonoid content (TFC) is crucial to comprehending their antioxidant potential.

The ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays are frequently used to evaluate the reducing power and free radical scavenging activities of plant extracts, respectively. According to Brand-Williams *et al.* (1995) and Benzie & Strain (1996), these tests offer important information on the antioxidant potential of natural compounds and their ability to fight oxidative stress. Additionally, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which gauges cell survival and proliferation in response to treatment, can be used to assess the anticancer activity of plant extracts (Mosmann, 1983). There is an urgent need to investigate natural substances with anticancer qualities that can supplement current treatment approaches, given the rising incidence of cancer globally and the shortcomings of traditional medicines.

In early research, the bioactive qualities of *Alpinia nigra* and *Hedychium coronarium* have showed promise. For example, because of its high concentration of flavonoids and phenolic compounds, *Alpinia nigra* has been shown to have anti-inflammatory, antibacterial, and antioxidant properties (Rout *et al*., 2009). Likewise, essential oils and extracts from *Hedychium coronarium* exhibit strong biological activity, demonstrating antibacterial, anti-inflammatory, and anticancer properties (Joshi *et al*., 2008). Nevertheless, there aren't many thorough investigations on these plants' anticancer and antioxidant properties, especially when considering Manipur's distinctive biodiversity. By assessing the antioxidant activity of the TPC, TFC, DPPH, and FRAP assays and the anticancer activity of the MTT assay in the rhizomes of *Alpinia nigra* and *Hedychium coronarium* from Manipur, this work seeks to close this gap.

Two well-characterized human cancer cell lines, HeLa (cervical adenocarcinoma) and A549 (lung adenocarcinoma), were used to evaluate the anticancer potential of these plant extracts. Lung cancer, one of the main causes of cancer-related deaths globally, is frequently studied using the A549 cell line as a model (Giard *et al*., 1973). Because of their resilience and reproducibility, HeLa cells—which are generated from cervical cancer—are among the most thoroughly researched cell lines in cancer research (Masters, 2002). These cell lines offer a trustworthy platform for assessing plant extracts' cytotoxic properties and capacity to stop the growth of cancer cells. A comprehensive understanding of the anticancer mechanisms of *Alpinia nigra* and *Hedychium coronarium* rhizomes, such as their capacity to trigger apoptosis, impede cell cycle progression, or alter signaling pathways implicated in the progression of cancer, is made possible by the use of these cell lines in the MTT assay (Freshney, 2010).

The results of this study should give a scientific foundation for the traditional use of Manipur's medicinal plants and add to the expanding body of knowledge on their therapeutic potential. Additionally, the discovery of bioactive substances with anti-cancer and antioxidant qualities may result in the creation of innovative pharmaceuticals and nutraceuticals, opening up new therapy and preventative options for cancer and disorders linked to oxidative stress. By connecting traditional wisdom with contemporary science, this study emphasizes how crucial it is to protect and utilize Manipur's abundant biodiversity for the sake of human health.

MATERIALS AND METHODS

A. Plant Sample Collection

During their prime growing season, fresh rhizomes of *Alpinia nigra* and *Hedychium coronarium* were gathered from the peripheral areas of the Loktak Lake, Manipur. Voucher specimens *Alpinia nigra* MU/ACC/1534 (Fig. 1) and *Hedychium coronarium* MU/ACC/1533 (Fig. 2) were placed in an herbarium for reference after the authenticity of the plant specimens was verified in the Department of Life Sciences (Botany), Manipur University. After being properly cleaned with distilled water to get rid of any dirt or debris, the rhizomes were allowed to shade dry at room temperature before being ground into a fine powder with a mechanical grinder. The powdered material was stored in airtight containers at 4°C until further use with labels PU for *Alpinia nigra* and LL for *Hedychium coronarium.*

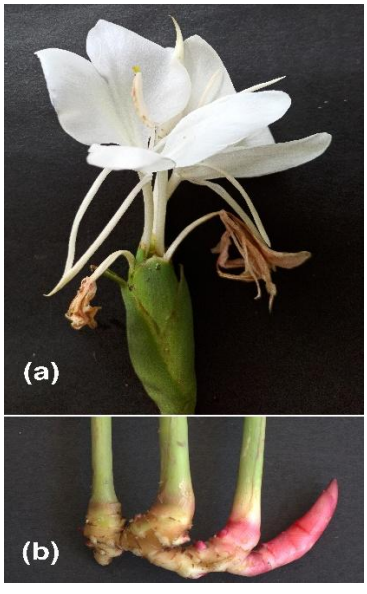

Fig. 1. *Alpinia nigra* (a) Inflorescence (b) Rhizome.

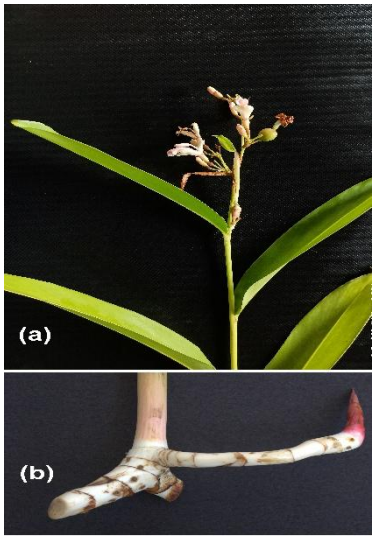

Fig. 2. *Hedychium coronarium* (a) Inflorescence (b) Rhizome.

B. Sample extract preparation

Methanol (AR grade) was used as the solvent for the Soxhlet extraction of the 100 g of the powdered rhizomes. After 6 to 8 hours of extraction, the solvent was removed using a hot plate set at 40°C. After lyophilizing the resultant crude extracts was kept at -20°C until additional examination (Adam *et al*., 2019). The yield is expressed as a percentage and is calculated using the following formula:

$$Extraction\ Yield\ (\%) = \left(\frac{Weight\ of\ Extracted\ Material}{Weight\ of\ Dry\ Sample}\right) \times 100$$

C. Determination of Total Phenolic Content (TPC)

The Folin-Ciocalteu method, as outlined by Singleton and Rossi (1965), with slight modifications was used to ascertain the extracts' total phenolic content. In short, 1.5 mL of 2N Folin-Ciocalteu reagent and 1 mL of the extract (1 mg/mL) were mixed and incubated for 5 minutes. 4 ml of sodium carbonate solution (75g/L) was added to the mixture. A microplate reader was used to measure the absorbance at 765 nm after the mixture had been incubated for 30 minutes at room temperature. The same microplate reader was used in all the following absorbance measurements. The results were reported as milligrams of gallic acid equivalents (GAE) per gram of extract, with gallic acid serving as the standard (Kamtekar *et al*., 2014; Attard, 2013).

D. Determination of Total Flavonoid Content (TFC)

The aluminum chloride colorimetric method was modified from the procedure reported by Woisky and Salatino (Woisky *et al*., 1998). The calibration curve was created using quercetin. Separately, 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate, and 2.8 mL of distilled water were combined with the diluted standard solutions (0.5 mL). A microplate reader was used to measure the reaction mixture's absorbance at 415 nm following a 30-minute incubation period at room temperature. In the blank, the same volume of distilled water was used in place of the 10% aluminum chloride. Likewise, as previously mentioned, 0.5 mL of methanolic sample extracts were treated with aluminum chloride to determine the flavonoid content (Chang *et al*., 2002).

E. Anti-oxidant activity

DPPH Radical Scavenging Assay. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to assess the extracts' capacity to scavenge free radicals (Brand-Williams *et al*., 1995). In short, 1 mL of the extract at different doses (10–500 µg/mL) was combined with 2 mL of a 0.1 mM DPPH solution that had been produced in methanol. After 30 minutes of dark incubation, the mixture's absorbance at 517 nm was measured. The positive control in this experiment was ascorbic acid. The following formula was used to determine the percentage of DPPH radical scavenging activity:

$$\text{DPPH Scavenging Activity}\ (\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

Where,

- $A_{control}$ = Absorbance of the DPPH solution without the sample (blank).
- A_{sample} = Absorbance of the DPPH solution with the sample.

Ferric Reducing Antioxidant Power (FRAP) Assay. The FRAP assay was carried out according to the protocol described by Benzie and Strain (1996). The FRAP reagent was made by mixing 20 mM ferric chloride, 10 ml TPTZ solution in 40 mM HCl and 300 mM acetate buffer (pH 3.6) in a 1:1:10 ratio. A 100 µL aliquot of plant extract (10–500 µg/mL) was combined with 3 mL of FRAP reagent, and the reaction mixture was then incubated for 30 minutes at 37°C. The absorbance was measured at 593 nm. Trolox was used as the standard, and the results were expressed in Trolox equivalents (TE) (Rajurkar *et al*., 2011).

F. Anti-Cancer Activity

Culture of Cells. A549 (Human lung adenocarcinoma) and HeLa (Human cervical carcinoma) cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified incubator at 37°C with 5% CO_2. Cells were maintained in 75 cm^2 tissue culture flasks and passaged every 2–3 days using 0.25% Trypsin-EDTA when they reached 80–90% confluence. The cell suspension was centrifuged at 300 × g for 5 minutes and the pellet was resuspended in fresh complete medium. Cell viability was assessed using trypan blue staining and a hemocytometer. For the MTT assay, cells were seeded into 96-well plates at a density of 5×10^3 cells/well in 100 µL of complete medium and allowed to adhere for 24 hours (Freshney, 2010).

MTT Assay for Cell Viability. The cytotoxicity of sample extract LL and PU was evaluated against A549 and HeLa cells using the MTT assay (Mosmann, 1983). Stock solutions of the samples were prepared in dimethyl sulfoxide and diluted in DMEM to achieve final concentrations ranging from 1 to 250 µg/mL. After 24 hours of cell adherence in 96-well plates, the medium was replaced with 100 µL of fresh medium containing the test samples at specified concentrations. Negative control wells contained only the medium with 0.1% DMSO and Plates were incubated for 24 hours at 37°C in a 5% CO_2 incubator. Following incubation, 10 µL of 5 mg/mL MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) prepared in PBS was added to each well and incubated for 4 hours at 37°C. The medium was carefully removed, and the resulting formazan crystals were dissolved in 100 µL of DMSO. The absorbance was measured at 570 nm using a microplate reader. The percentage of cell viability was calculated using the formula:

$$Cell\,Viability\ (\%) = \left(\frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}}\right) \times 100$$

Where:

- A_{sample} = Absorbance of the treated cells (with the test compound or extract).
- $A_{control}$ = Absorbance of the untreated cells (control group).
- A_{blank} = Absorbance of the background (medium without cells).

Each concentration was tested in triplicate, and results were expressed as a percentage of cell viability relative to untreated control cells. The IC_{50} values for each sample were determined using GraphPad Prism (software version 8.4.2).

G. Statistical Analysis

Every experiment was carried out in triplicate, and the mean ± standard deviation (SD) was used to express the results. Group means were compared using Tukey's post hoc test after one-way analysis of variance (ANOVA). A p-value of <0.05 was considered statistically significant (GraphPad Prism, version 8.4.2).

RESULTS

A. Soxhlet Extraction Yield

The extraction yield is a measure of the solvent efficiency to extract specific components from the original material. They are 4.79 and 16.08 respectively (Table 1).

Table 1: Percentage yield of soxhlet extraction of *Alpinia nigra* (PU) and *Hedychium coronarium* (LL).

Sample	Percentage Yield (%)
LL (*Hedychium coronarium*)	16.08
PU (*Alpinia nigra*)	4.79

B. TPC and TFC

In this study, the rhizomes of both PU and LL showed significant amounts of phenolics and flavonoids. It may also be noted that methanol extracts more phenolic and flavonoid compounds as compared to other solvents. According to the correlation coefficient, the TPC and TFC of PU was 132.75 ± 0.37 (mg GAE/100g) and 67.28 ± 1.58 (mgQE/100g) and that of LL was 83.57 ± 0.72 (mg GAE/100g) and 67.28 ± 1.58 (mgQE/100g) respectively (Table 2).

C. Anti-oxidant activity

Two reliable assays, DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing antioxidant power) were used to assess antioxidant activity. The DPPH assay shows that PU had a lower IC50 value which was 103.74 ± 2.54 µg/ml than LL with 373.15 ± 5.19 µg/ml respectively (Fig. 3), suggesting a greater capacity to squelch free radicals.

Table 2: Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of *Alpinia nigra* (PU) and *Hedychium coronarium* (LL).

Sample	Total Phenolic Content (mgGAE/100g)	Total Flavonoid Content (mgQE/100g)
LL (*Hedychium coronarium*)	83.57 ± 0.72	46.26 ± 2.02
PU (*Alpinia nigra*)	132.75 ± 0.37	67.28 ± 1.58

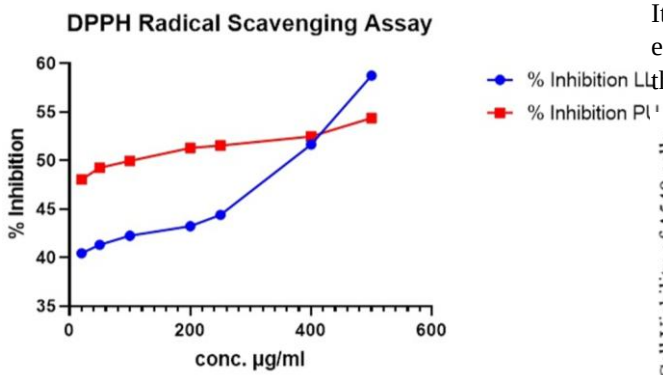

Fig. 3. DPPH Radical Scavenging Assay of *Alpinia nigra* (PU) and *Hedychium coronarium* (LL).

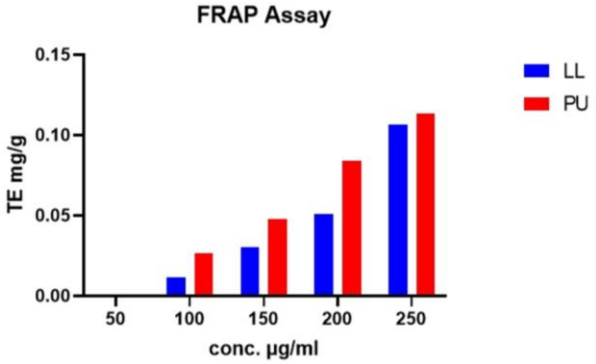

Fig. 4. FRAP Assay of *Alpinia nigra* (PU) and *Hedychium coronarium* (LL).

The FRAP assay provided additional support with *Alpinia nigra* exhibiting higher antioxidant reaching 0.1133 ± 0.007 TE mg/g at 250 µg/mL over *Hedychium coronarium* with 0.1064 ± 0.0046 TE mg/g at 250 µg/mL (Fig. 4).

D. Anti-cancer activity

The effect of the samples LL and PU on A549 and HeLa cells was examined for 24 h using the MTT test at various doses 1–250 µg/ml. The results showed a substantial ($p < 0.05$) difference between untreated and treated cells at various doses (Fig. 3, 4). At 10 µg/ml and above, PU significantly inhibited the growth of A549 and HeLa cells ($p < 0.05$); further concentrations of PU exerted gradient cytotoxic activity with increasing concentration; viability began to drop significantly ($p < 0.05$) at 100 and 250 µg/ml. Furthermore, PU showed higher potential anticancer activity and cytotoxicity in both the A549 and HeLa cells than LL, with IC50 values of 25.95 ± 1.98 and 32.12 ± 2.27 µg/ml respectively. Paclitaxel (Taxol) is taken as the standard reference for the IC50 values. The IC50 values of LL for A549 and HeLa cells are 202.62 ± 11.32 and 274.19 ± 13.85 µg/ml respectively (Table 3).

It also indicates the both the samples LL and PU exhibits better anti-cancer activity in the A549 cells than the HeLa cells.

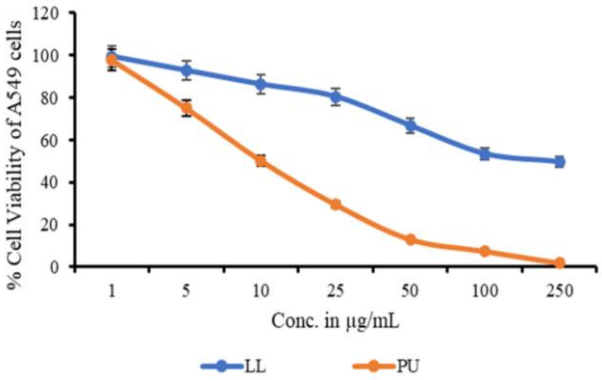

Fig. 5. Concentration-dependent cytotoxic effects of *Alpinia nigra* (PU) and *Hedychium coronarium* (LL) against A549 cells, assessed using the MTT assay. Data are presented as mean ± standard deviation (n = 3). PU exhibited significantly higher cytotoxicity compared to LL at concentrations of 10 µg/mL and above ($p < 0.05$). Statistical significance was determined using one-way ANOVA test.

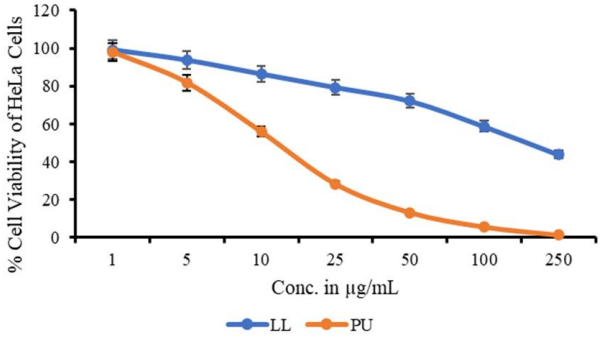

Fig. 6. Cytotoxicity of *Alpinia nigra* (PU) and *Hedychium coronarium* (LL) against HeLa cells at varying concentrations (1–250 µg/mL), evaluated via the MTT assay. Data are represented as mean ± SD (n = 3). PU showed significantly higher cytotoxicity compared to LL at concentrations ≥10 µg/mL ($p < 0.05$), determined by one-way ANOVA test.

Table 3: Comparison of IC50 values (in µg/mL) of *Alpinia nigra* (PU) and *Hedychium coronarium* (LL) against A549 and HeLa cells.

Samples	IC50 against A549 cells	IC50 against HeLa Cells
LL	202.62 ± 11.32	274.19 ± 13.85
PU	25.95 ± 1.98	32.12 ± 2.27

DISCUSSION

This study examined the anticancer and antioxidant properties of the rhizomes of two medicinal plants that

are traditionally utilized in Manipur, India: *Hedychium coronarium* (Fig. 2) and *Alpinia nigra* (Fig. 1). Strong antioxidant qualities were indicated by both plants' high total phenolic content (TPC), total flavonoid content (TFC), and potent capacity to scavenge radicals (Table 2). The extracts showed potential as natural anticancer agents by demonstrating dose-dependent cytotoxicity against cervical adenocarcinoma (HeLa) and lung adenocarcinoma (A549) cell lines (Table 3). These results offer a biological basis for their therapeutic effectiveness and validate their ethnomedical uses in northeastern India.

A. Antioxidant Activity

DPPH and FRAP assays, which are commonly used techniques for evaluating free radical scavenging and reducing potential, were used to investigate the antioxidant properties of *Alpinia nigra* and *Hedychium coronarium* rhizomes (Prior *et al*., 2005; Apak *et al*., 2007). The elevated TPC and TFC values imply that flavonoids and phenolics are essential for antioxidant action. By donating electrons or hydrogen atoms, these substances can scavenge reactive oxygen species (ROS) and stop oxidative damage (Kähkönen *et al*., 1999).

Because of its increased phenolic content, *Alpinia nigra* outperformed *Hedychium coronarium* in the DPPH experiment (Fig. 3), which demonstrated considerable radical scavenging action. Both extracts' significant reducing activity was also validated by the FRAP assay (Fig. 4), suggesting that they may be able to donate electrons to counteract oxidative stress. These findings are in line with earlier research that documented the antioxidant capacity of Hedychium and Alpinia species (Peng *et al*., 2022). The antioxidant properties of these plants may help prevent the start or progression of chronic diseases like cancer, cardiovascular problems, and neurodegeneration, as oxidative stress plays a key role in these ailments (Pham-Huy *et al.,* 2008). Notably, by lowering oxidative DNA damage and modifying redox-sensitive signaling pathways, antioxidants are also associated with the prevention of cancer (Pisoschi & Pop 2015).

B. Anticancer Activity

Both plant extracts demonstrated dose-dependent cytotoxicity against A549 and HeLa cells, according to the MTT experiment (Fig. 5). Perhaps as a result of its stronger proportion of bioactive phenolics and flavonoids, *Alpinia nigra* (Fig. 1) exhibited comparatively higher activity. These substances have been shown to disrupt cancer-related signaling pathways, alter cell cycle progression, and trigger apoptosis (Sak, 2012; Middleton *et al*., 2000).

One common model for researching lung adenocarcinoma, a major source of cancer-related fatalities globally, is the A549 cell line (Gazdar *et al*., 2010). The extracts' potential for treating lung cancer is suggested by their cytotoxic action on A549 cells. Similarly, because of their resilience and repeatability, HeLa cells—which are generated from cervical carcinoma—continue to be one of the most researched cancer models (Masters, 2002). The extracts' significant cytotoxicity toward HeLa cells demonstrates its use in the treatment of cervical cancer (Fig. 6).

According to Kooti *et al.* (2017), the anticancer effects most likely entail a number of processes, such as the regulation of cyclin-CDK complexes involved in cell proliferation, the activation of mitochondrial apoptosis pathways, and the reduction of anti-apoptotic proteins like Bcl-2. Furthermore, by suppressing the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs), polyphenols may prevent tumor spread (Kopustinskiene *et al.*, 2020).

C. Implications for Traditional Medicine

The findings support the traditional ethnomedical usage of *Hedychium coronarium* and *Alpinia nigra* across Manipur. Their application in the treatment of cancer and oxidative stress-related disorders is scientifically validated by the documented biological actions. The discovery of flavonoids and phenolics as important bioactive agents further emphasizes the medicinal benefits of the native flora and the significance of protecting indigenous knowledge and biodiversity (Fabricant & Farnsworth 2001). Novel phytotherapeutics and nutraceuticals that are intended to prevent or manage diseases with fewer adverse effects than synthetic medications can be created by combining scientific validation with ethnopharmacological knowledge.

CONCLUSION

In summary, this study showed that the high phenolic and flavonoid content of the rhizomes of *Hedychium coronarium* and *Alpinia nigra* confers notable antioxidant and anticancer activities. These plants may be used as natural sources of medicinal drugs due to their significant antioxidant potential and dose-dependent cytotoxic effects on A549 and HeLa cells. These findings support their long-standing medical application and demonstrate their potential for creating novel therapies for conditions linked to oxidative stress and cancer. To identify active ingredients, elucidate mechanisms of action, and assess in vivo safety and efficacy, more investigation is required.

FUTURE SCOPE

The result of this study establishes a base for further examination of their anticancer potential. Further identification and definition of the bioactive compounds responsible for the effects, followed by a thorough mechanistic analysis of cell cycle arrest, apoptosis and ROS-mediated pathways is necessary. After confirming in vivo validation of therapeutic relevance, their application can further be enhanced by exploring synergistic interactions. Thereby, paving the way for the creation of Phyto therapeutic anticancer drugs from these rhizomes that are clinically valid.

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