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Anticancer Screening of Few Wild Edible Fruits in Mizoram, Northeast India using MTT Assay

Rody Ngurthankhumi[1], T.K. Hazarika[1], Esther Lalruatsangi[2], H. Lalhmachhuani[1], Panthor Debbarma[1], Zothansiama[3]

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ABSTRACT

Background: Cancer is an assortment of diseases resulting from disrupting cell cycle regulation. It is characterised by the proliferation of cells in an unregulated manner. The development of cancer can be attributed to a variety of factors, both external and internal. Various plants are rich in a variety of bioactive phytochemicals and essential nutrients. Several studies over the past few decades have shed light on the significant role of these phytochemicals in preventing chronic diseases such as cancer, diabetes and coronary heart disease. The MTT *in vitro* cell proliferation assay known for its high reliability and commonly used to measure whole-cell cytotoxicity across various cell lines is a commonly employed method for assessing the initial effectiveness of synthetic derivatives and natural products in combating cancer.

Methods: For the current study wild edible fruits were collected from different regions of Mizoram, northeast India during 2020-2021. The study involved conducting an MTT *in vitro* cell proliferation assay on A549 cell lines using methanolic extract of seven wild edible fruits.

Result: The methanolic extract of the seven wild edible fruits exhibited a significant cytotoxic effect on the A549 cell lines. *Tamarindus indica* demonstrated the lowest IC_{50} value of 119.77±0.20 µg/ml indicating its high cytotoxicity against A549 cell lines, followed by *Citrus grandis* with an IC_{50} value of 176.63±10.66 µg/ml among the seven wild edible fruits studied.

Key words: Cancer, Cell lines, Cytotoxic, *In vitro,* MTT, Wild edible fruits.

INTRODUCTION

Cancer is a prominent contributor to mortality rates and a significant obstacle to improving life expectancy worldwide. Cancer is an assortment of disorders marked by various genetic and cellular abnormalities that lead to unrestrained cell proliferation, invasion and progression (Hanahan and Weinberg, 2000). According to GLOBOCON 2020, data were obtained from 185 countries encompassing 36 different forms of malignancies and there were about 19.3 million newly diagnosed cases of cancer and 10 million deaths caused by cancer globally. In 2020, it is anticipated that Asia accounts for half of all cancer diagnoses and 58.3% of cancer-related deaths worldwide. This is significant since Asia is home to 59.5% of the global population. Europe comprises 22.8% of the overall cancer cases and 19.6% of the cancer-related fatalities, although having only 9.7% of the world's population. America, on the other hand, provides 20.9% of the total cancer cases and 14.2% of the global cancer mortality (Sung *et al*., 2021). According to projections, the number of new cancer cases is expected to reach 26 million annually by 2030, resulting in 17 million deaths from cancer each year (Thun *et al*., 2010).

Although all types of cancer pose a tremendous health fatality, among them, lung cancer is a highly fatal form of cancer that has a significant impact on both rates of morbidity and mortality (Nghakliana *et al*., 2021). Its high mortality and poor prognosis are due to the difficulty of early diagnosis and the high potential to invade locally and metastasise to distant organs (Hsieh *et al*., 2015). Lung cancer accounted for 1.6 million deaths globally in relation to all cancer-related fatalities (Torre *et al*., 2015). The A549 cell line comprises hypotriploid alveolar basal epithelial cells. The establishment of this cell line was first accomplished by Giard *et al.* (1973) by the extraction and cultivation of lung carcinoma tissue from the excised tumour of a 58-year-old Caucasian man. These cells proliferate as a single layer in a laboratory setting and are often used to evaluate the anticancer effects of various plant extracts *in vitro* (An *et al*., 2014; Venugopal *et al*., 2017; Kumar *et al.,* 2017).

Though there are several cancer treatment options available, the overall death rate for cancer does not seem to diminish. Over the last decade, recently created synthetic

[1]Department of Horticulture, Aromatic and Medicinal Plants, Mizoram University, Aizawl-796 004, Mizoram, India.
[2]College of Horticulture, Central Agricultural University, Thenzawl-796 186, Mizoram, India.
[3]Department of Zoology, Mizoram University, Aizawl-796 004, Mizoram, India.

Corresponding Author: T.K. Hazarika, Department of Horticulture, Aromatic and Medicinal Plants, Mizoram University, Aizawl-796 004, Mizoram, India. Email: tridip28@gmail.com

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chemotherapeutic medicines, which are now being used in clinical environments, have not lived up to expectations despite the substantial expenditure in their development. Therefore, there is a continuous need to develop innovative, powerful and affordable anti-cancer drugs (Coseri, 2009). Plants and natural products remain a significant reservoir of anti-cancer substances because of their safety, effectiveness and reduced adverse effects. Since the beginning of ancient medicine, plant-derived chemical substances have been used to treat human ailments. Over the last three decades, there has been a growing interest in natural products due to their potential as innovative agents for preventing and treating cancer (Newman, 2008; Newman *et al.,* 2003). There have been reports of over 3000 plants from around the world possessing properties that may be effective against cancer. On a global scale, the utilisation of plant-derived products for cancer treatment ranges from 10% to 40%, increasing to 50% among patients in Asia (Solowey *et al.,* 2014).

The MTT in vitro cell proliferation assay is a commonly utilised method for assessing the initial anticancer potential of both natural and synthetic derivatives (Janice 2013). The measurement of viable cells is conducted using colourimetry, which is based on the principle of mitochondrial dehydrogenase enzymes. These enzymes produce NADH or NADPH, reducing colourless 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide salt and turning it into a visually detectable soluble formazan product. This reaction occurs due to the mitochondrial activity of viable cells at a temperature of 37°C. The amount of the coloured product is directly linked to the number of live cells in the culture. This is because the MTT reagent can only be reduced to formazan by metabolically active cells (Pallaka *et al.,* 2019).

Keeping all the above information in mind, the present investigation has been intended to investigate the anti-cancer properties of seven wild edible fruits, *viz*. *Citrus grandis, Citrus jambhiri, Citrus medica, Morus nigra, Rubus treutleri, Artocarpus heterophyllus* and *Tamarindus indica* of Mizoram, India. This study may be useful to know the best wild edible fruits having anti-cancerous properties and help the pharmaceutical companies to formulate the anti-cancerous drugs by using these naturally occurring wild edible fruits.

MATERIALS AND METHODS

The preent investigation was carried out at Mizoram University, Aizawl, Mizoram, India during during 2021 to 2022 by collecting seven distinct wild edible fruits was gathered from four districts namely Aizawl, Khawzawl, Serchhip and Lunglei of Mizoram, North-east India.

Source of reagents

Trypsin, 3-4, 5-dimethylthiazole-2-yl-2, 5-diphenyl tetrazolium bromide (MTT), Fetal Bovine Serum (FBS) and Dimethyl sulfoxide (DMSO) were purchased from Hi-Media Laboratories Pvt., Ltd. (Mumbai, India).

Preparation of fruits extracts

Seven wild edible fruits, *viz*. *Citrus grandis, Citrus jambhiri, Citrus medica, Morus nigra, Rubus treutleri, Artocarpus heterophyllus* and *Tamarindus indica* were gathered from several regions in Mizoram, India. The fruits were cleaned, cut, air-dried and ground into powder. The crushed fruits were defatted with petroleum ether in a Soxhlet apparatus at 40°C for 30 cycles and dried at 40°C overnight to eliminate any remaining petroleum ether. The powdered material underwent further extraction with methanol using the Soxhlet device for at least 40 cycles. The liquid extracts were filtered and concentrated using a rotary evaporator (Buchi, Germany) at 40°C under reduced pressure for about 5 hours and then freeze-dried.

Cell lines and culture

Type II human lung adenocarcinoma cell line (A549 cells) was procured from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were maintained in MEM supplemented with 10% FBS and 1% L-Glutamine in a humidified incubator with 5% CO_2 at 37°C (Eppendorf, Hamburg, Germany).

Cytotoxicity assay (MTT assay)

The cytotoxicity of various fruit extracts was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction test described by Mossman (1983). 100 µl of MEM with 1×10^4 cells were placed into 96-well plates. The cells were exposed to various doses of different fruit extracts (ranging from 5 to 500 µg/ml) for 24 hours, after a 24-hour adhesion period at 37°C and 5% CO_2, alongside a control sample. Medium-containing extracts were removed and cells were washed with medium without FBS after the treatments. Next, cells were exposed to 10 µl of MTT (5 mg/ml) and incubated for 2 hours at 37°C in a CO2 incubator. Subsequently, 100 µl of DMSO was used to dissolve the insoluble purple formazan crystals. Following a 30-minute incubation period, the solution's absorbance was assessed at 560 nm using a microplate reader (Spectramax m2e, Molecular Devices). Three separate trials with three replicates were performed for each treatment. Cytotoxicity was quantified as the percentage of inhibition using the following formula:

$$\% \text{ inhibition} = \frac{\text{Control-Treatment}}{\text{Control}} \times 100$$

Statistical analysis

All data were expressed as mean±standard error of the mean. One-way ANOVA followed by Tukey's test was performed to test significant variations between control and treatment groups. SPSS ver.25.0 software (SPSS Inc, Chicago, Illinois, USA) and Graph Pad Prism ver. 8.0 was used for statistical and graphical analyses. A p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Historically, various plants have been utilised in indigenous medicine for their natural healing properties. These plants have demonstrated practical therapeutic benefits, such as cardiovascular disease prevention, anti-inflammatory responses, antimicrobial properties and anticancer activity. Several anticancer agents have been sourced from them, such as taxol, vinblastine, vincristine, etoposide and more. Natural remedies are both more effective and safer than synthetic drugs, with fewer side effects (Gullett *et al.,* 2010). Presently, the existing radiotherapy and chemotherapeutic treatments are successful in addressing cancer, yet they offer minimal advantages to patients (Soengas and Lowe, 2003). The rise of resistance to cancer chemotherapy has prompted researchers to explore natural products from plants and marine sources. Hence, it is crucial to identify and cultivate alternative therapeutic agents. It is now widely acknowledged that the positive impacts of plants on health are a result of the intricate combination of compounds found in the entire plant rather than individual components alone. Many plant-derived compounds are undergoing thorough examination for their potential anticancer properties (Nghakliana *et al.,* 2021).

Recent research has concentrated on using medications from conventional medicine to improve cancer therapy. Although there are many claims on the therapeutic properties of various wild edible fruits such as *Citrus grandis, Citrus jambhiri, Citrus medica, Morus nigra, Rubus treutleri, Artocarpus heterophyllous* and *Tamarindus indica,* research on their potential for cancer therapy remains scarce (Abu Bakar *et al.,* 2016; Angami *et al.,* 2024; Kanfon *et al.,* 2023; Pan *et al.,* 2023; Silva *et al.,* 2020). This research aimed to investigate these fruits' growth inhibitory and cytotoxic effects on human lung adenocarcinoma A549 cells. The objective of targeting cell proliferation in cancer is to trigger cell death or halt the cell cycle by using cytotoxic chemicals. The MTT assay is a quick and established technique used to assess the cytotoxicity of medicines in different cultured cells, where the decrease of MTT only happens in metabolically active cells.

To determine the cytotoxic effect of methanolic extracts of different fruits, A549 cells were treated with varying doses

Table 1: Evaluation of IC_{50} values of the seven samples.

Sample name	Concentration (µg/ml)	O.D at 560 nm	% inhibition	IC_{50} (µg/ml)
Control	0	0.775	0	-
Citrus grandis	50	0.686	11.53	176.63
	100	0.659	14.92	
	200	0.238	69.25	
	300	0.207	73.33	
Citrus jambhiri	100	0.696	12.74	659.47
	200	0.680	14.79	
	300	0.617	22.68	
	400	0.495	37.93	
Citrus medica	100	0.685	14.20	491.63
	200	0.643	19.42	
	300	0.612	23.31	
	400	0.394	50.58	
Morus nigra	50	0.898	6.56	527.87
	100	0.872	9.30	
	200	0.695	27.64	
	300	0.653	32.08	
Rubus treutleri	50	0.834	13.22	236.70
	100	0.768	20.12	
	200	0.679	29.34	
	300	0.286	70.27	
Artocarpus heterophyllus	50	1.183	11.63	2074.33
	100	1.121	16.28	
	200	1.096	18.17	
	300	0.999	25.42	
Tamarindus indica	50	0.915	19.14	119.77
	100	0.605	46.525	
	200	0.320	71.702	
	300	0.315	72.203	

for 24 hr. The inhibition (%) of A549 cells by methanolic extracts of different wild edible fruits was plotted against log doses to calculate IC_{50} (Fig 1). Among the various extracts of drupes, from Table 1, *Tamarindus indica* extract was found to possess the highest cytotoxicity with an IC_{50} of 119.77±0.20 µg/ml, followed by *Citrus grandis* extract (IC_{50} = 176.63±10.66 µg/ml), *Rubus treutleri* extract (IC_{50} = 236.70±6.49 µg/ml), *Citrus medica* extract (IC_{50} = 491.63±61.87 µg/ml), *Morus nigra* extract (IC_{50} = 527.87±4.40 µg/ml), *Citrus jambhiri* extract (IC_{50} = 659.47±42.55 µg/ml) and *Artocarpus heterophyllus* extract (IC_{50} = 2074.33±274.85 µg/ml). However, no significant statistical variation was observed in the cytotoxic effects among *Tamarindus indica, Citrus grandis* and *Rubus treutleri* extracts. Furthermore, the statistical analysis showed that *Citrus grandis, Citrus medica, Rubus treutleri and Morus nigra* were statistically at par while *Citrus jambhiri* and *Artocarpus heterophyllus* were significantly different from the rest of the samples (Fig 2). A549 cells treated with the methanolic extract of the fruit as indicated in the study, leading to a dose-dependent rise in cytotoxicity as obtained in the results. The cytotoxic actions of these extracts can be attributed to the presence of certain phytochemicals found in the fruits such as phenols, flavonoids, alkaloids and antioxidants as reported in various other research (Abu Bakar *et al.,* 2016; Angami *et al.,* 2024; Kanfon *et al.,* 2023; Pan *et al.,* 2023; Silva *et al.,* 2020).

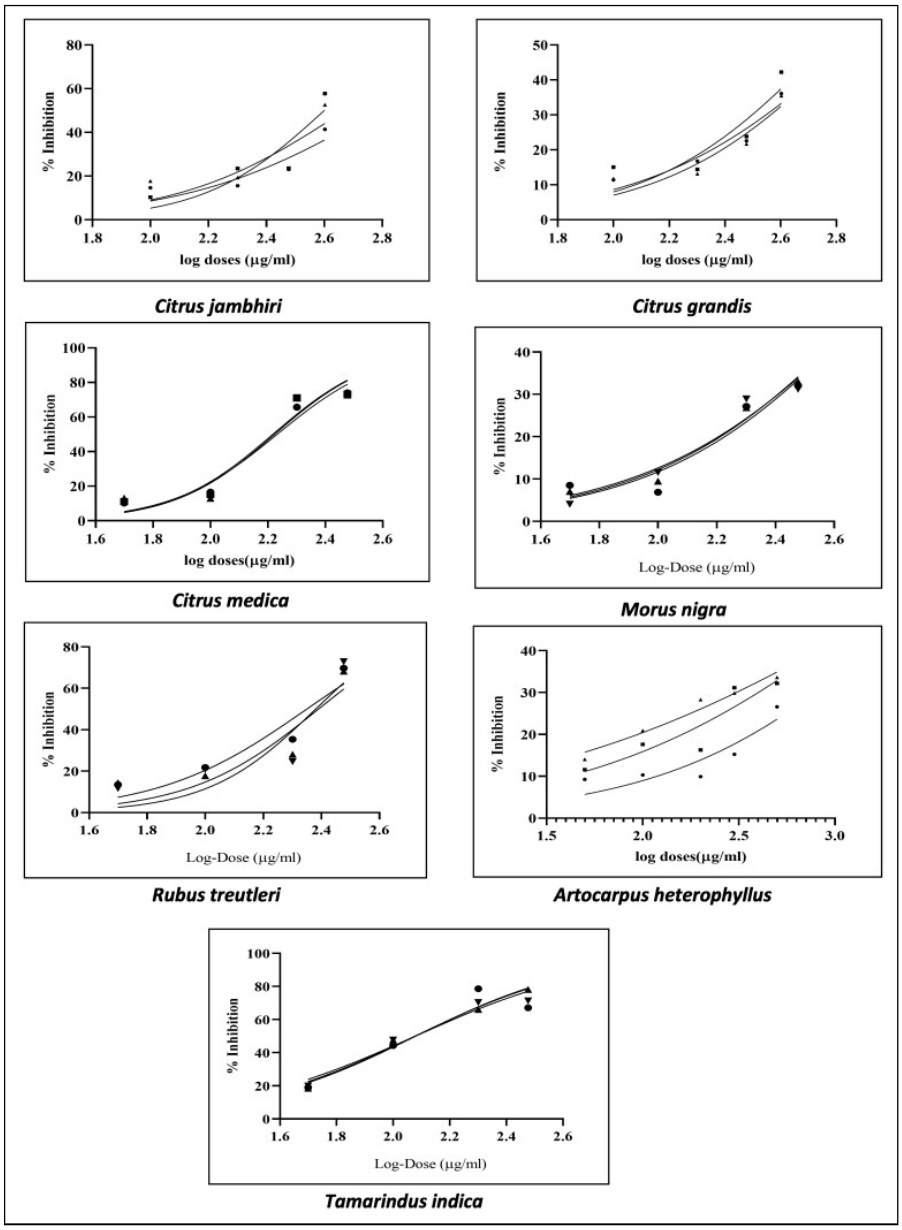
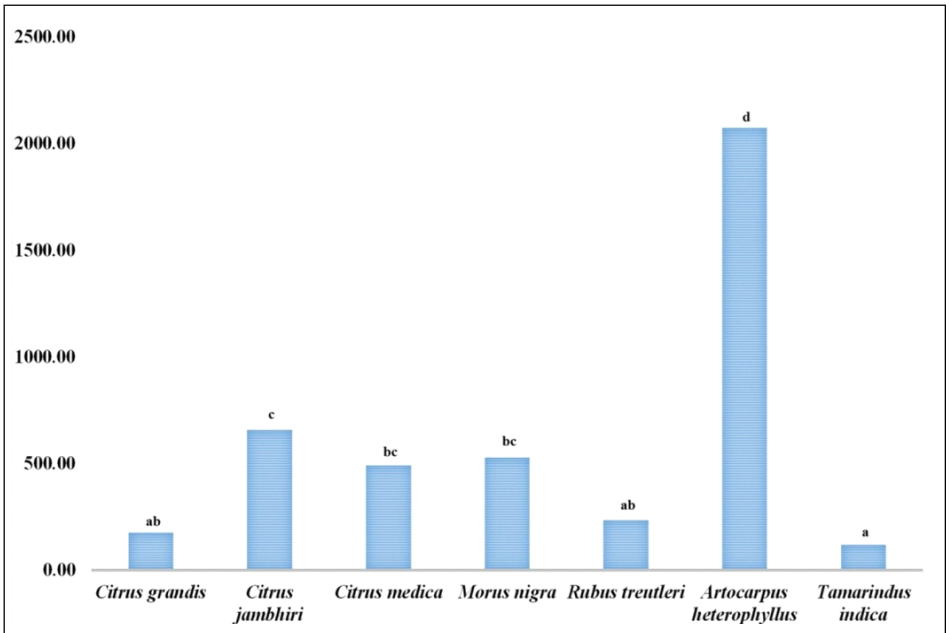


Fig 1: Plots of log doses of fruit extracts against inhibition (%) for calculating IC_{50}.



*Values with different lower-case superscript ($^{a-d}$) letters in a column are significantly different among the fruits at $p < 0.05$ (DMRT test performed for separation of mean). Values are expressed as mean ± SD with three replications (n = 3) for each experiment.

Fig 2: Comparison between IC_{50} values of the seven wild edible fruits.

CONCLUSION

The results of our study indicate that the seven wild edible fruits exhibited cytotoxic effect on A549 lung cancer cells. Among the studied wild edible fruits, *Tamarindus indica, Citrus grandis* and *Rubus treutleri* have shown strong anticancerous properties and may be ptotentially utilised for the development of herbal formulations to treat lung cancer. From the results of the present investigation, it can be recommended to carry out a bioassay-guided fractionation study in these fruits to isolate and characterise the active ingredients responsible for its anti-cancer properties.

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Conflict of interest

The authors declare that there is no conflict of interest in whatsoever.

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