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*Research Article*

# Anti-Cancer Activity of the Fruit Extract of *Zanthoxylum Rhetsa* in Human HepG2 and U87MG Cancer Cells

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**ABSTRACT**
*Zanthoxylum rhetsa* is a traditional medicinal plant species used locally amongst the inhabitants of Northeast India, owing to its anti-septic, anti-bacterial, and anti-diabetic properties. Various reports have suggested that the occurrence of diverse secondary metabolites may be accountable for the medicinal activities. In this study, we have examined the fruit of *Z.rhetsa* to identify the secondary metabolites present and examine their in vitro anticancer activity in human liver HepG2 and glioblastoma U87MG cancer cell lines. Chemical profiling was carried out by Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography Mass Spectroscopy (GC-MS) techniques in the ethanolic fruit extract. Additionally, molecular docking was performed with the compounds from GC-MS profiling to assess their interaction with the epidermal growth factor receptor (EGFR) protein. Phytochemical screening of *Z.rhetsa* fruit showed remarkable anti-oxidant activity and the presence of alkaloids, flavonoids, and phenols in it. The findings demonstrated that the extracts exhibited a dose-dependent anticancer activity on HepG2 and U87MG cell lines, with ethanolic extract demonstrating the highest potency. Experimental findings also revealed the apoptotic activity of the fruit extract via DNA fragmentation and a live dead assay and antiproliferative effect, as seen by a remarkable decrease in colony formation of the cells. Chemical profiling by GC-MS analysis in the ethanolic fruit extract demonstrated the presence of Nerolidol, a compound previously reported for its cytotoxicity against cancer cells. A molecular docking study of Nerolidol and EGFR demonstrated low binding energy in a protein-ligand complex. Our study reported the cytotoxic activity of ethanolic fruit extract of *Zanthoxylum rhetsa* potentially attributed to several secondary metabolites.

**Keywords:** *Zanthoxylum rhetsa*; secondary metabolites; HepG2; U87MG; GC-MS; FTIR.

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## INTRODUCTION:

Cancer, a pathological disease that promotes uncontrolled cell growth and harms the human body, has drastically risen in recent years. A 2020 study reported that cancer has killed over 10 million people worldwide. Certain behavioral patterns such as reduced fruit intake, sedentary lifestyle, lack of physical activity, and alcohol and cigarette consumption have contributed to approximately 1/3 of cancer-related mortalities[1]. Nature offers a very effective and dependable reservoir of novel

medications, including anticancer compounds. Around 70% of current anticancer medicines are derived from plants[2].

*Zanthoxylum rhetsa* (Roxb.) DC, commonly referred to as *Zanthoxylum budrunga*, belonging to the Rutaceae family, is an aromatic tree of medium size characterized by cone-shaped thorns on the trunk and branches. This species is found in tropical and subtropical areas, particularly Bangladesh, China, India, Indonesia, and Malaysia[3]. The plant species is traditionally believed to possess diverse therapeutic properties. It is commonly used amongst the Naga tribes of India as a folklore medicine against deworming. The plant's stem and root bark are used as a remedy against maladies such as rheumatism, malaria, and loss of stomach tone[4]. In the conventional approach, diverse components of the plant are employed for their aromatic, astringent, anti-microbial, anti-septic, and anti-diabetic properties.

Moreover, *Z. rhetsa* is also reported to be an effective remedy against cholera, snake bites, toothache, and inflammatory skin diseases[5, 6]. Previous study has reported the presence of phyto-constituents such as xanthyletin, sesamin; 3, 5-dimethoxy-4-geranyloxycinnamyl alcohol; zanthorhetsamide and 8-methoxy-N-methylflindersine belonging to monolignols, coumarins, alkaloids and lignans class of compounds[7, 8]. Furthermore, analysis of the root bark reported the presence of chelerybulgarine, 21-episimulanoquinoline, 2, 11-didemethoxyvepridimerine B, and rhetsidimerine as phyto-constituents belonging to quinolones, terpenes, and alkaloids class of compounds[9]. In a recent investigation, the antioxidant potential of *Z. rhetsa* seeds was examined, with a particular focus on its concentration-dependent behavior[10]. Another study undertaken by Kumar GR et al. on various components of the plant's fruit revealed that the seed coat and the whole fruit exhibited the most substantial quantities of total phenol content and displayed the highest levels of antioxidant activity[11]. Chureeporon et al., in their study, demonstrated that *Z. rhetsa* alleviated pain and decreased inflammation by inhibiting the production of pro-inflammatory markers, such as TNF-α, NO, and PGE2 in RAW264.7 macrophages[12]. Yet another study by Santhanam RK et al. demonstrated that the chloroform fraction from the bark of *Z. rhetsa* possesses significant bioactivity and successfully isolated and characterized two compounds, yangambin and kobusin, which are tetrahydrofuran lignans columbamine, a berberine alkaloid, and lupeol, which is a triterpenoid. The cytotoxic activity was conducted on two cell lines: human dermal fibroblasts (HDF) and mouse melanoma (B16-F10). Results showed no cytotoxicity by the crude, fractions, and isolated compounds on the HDF cell line. In contrast, isolated compounds such as koubusin, columbamine, lupeol, and yangambin effectively demonstrated cytotoxicity against the B16-F1O melanoma cell line. The results suggested that bioactivity is mainly due to lignin and alkaloids in the *Z. rhetsa* bark[13].

Alkaloids, widely known to possess cytotoxic activity, are also present in various medicinal plants. Even, *Z. rhetsa* reported the presence of several alkaloids such as Skimmianine, Dihydrochelerythrine, quinazoline-6-carboxylic acid, 1- methoxy-7, 8-dehydrorutaecarpine and arnottianamide. Of these, the alkaloid compound 6-acetonyldihydro-chelerythrine possesses both anti-oxidant and anti-HIV activity[14]. Terpinene and Sabinene, two essential oils extracted from *Z.rhetsa*, demonstrated anti-cancer activity on breast cancer cells, thereby suggesting the beneficial role of the essential oils as an anti-proliferative and cell survival agent[15].
The cytotoxic activity of *Z. rhetsa* fruits from the Northeastern part of India has yet to be scientifically validated. The findings of our inquiry into the cytotoxic potential and preliminary phytochemical screening of *Z. rhetsa* fruits are presented in this study.

## MATERIALS AND METHODS:

### Sample collection, authentication, and extraction of *Z. rhetsa* (Roxb.) DC:

*Z. rhetsa* (Roxb.) DC fruits were collected during the month of August 2020 from Sonapur tea estate, Assam, India (26.1177°N, 91.9717°E). The authentication of the samples was carried out at the Botanical Survey of India, Shillong, Meghalaya, and the voucher specimen (BSI/ERC/Tech/2020/1315) has been submitted. 20g of cleaned and shade-dried fruits of *Z. rhetsa* were coarsely grounded and extracted exhaustively with various solvents like water, methanol, ethanol, chloroform, and ethyl acetate for 48 hours. The water extract was filtered and further lyophilized, and the other extracts were concentrated using a Rotavapor and stored at -20°C until further screened for biological activity. The extract yields were measured by the following equation.

$$\%\text{yield} = \frac{weightofdriedextract}{weightofpowderfruit} \times 100$$

### Phytochemical screening of the extracts of *Z. rhetsa* (Roxb.) DC:

The phytochemical investigation was carried out for all the extracts for the detection of alkaloids, anthraquinone glycosides, saponins, flavonoids, phenols, steroids, terpenoids, amino acids, proteins, carbohydrates, tannins, and fixed oil and fats. The test followed for each of the phytochemical screened are enlisted in Table 1.

### Estimation of total alkaloids, flavonoids, and phenolic content of the fruit of *Z. rhetsa* (Roxb.) DC:

***Alkaloids****:* Total alkaloid content was measured via spectrophotometric analysis utilizing the Bromocresol green method. Atropine (MERCK, A0132), was employed as the standard compound for calibration purposes[16].
***Flavonoids***: The colorimetric Aluminum chloride technique quantified the total flavonoid concentration. Quercetin (MERCK, Q4951), was used as the standard reference[17].
***Phenol****:* The total phenolic content was quantified using the Folin-Ciocalteu technique[18].
*In-vitro* antioxidant activity:

The *in vitro* antioxidant activity of *Z. rhetsa* fruits was measured via DPPH radical scavenging assay[18]. The optical density was calculated at a wavelength of 517 nm in a multimode plate reader (BIOTEK, SYNERGY H1). Ascorbic acid was the reference standard for the experimental technique. The $IC_{50}$ was calculated as follows.

$IC_{50} = (1-□_1/□_0) \times 100$,
$□_0$= Absorbance of the control
$□_1$= Absorbance of the extract or standard.

**Cell culture studies:**
**Cell culture:**
Human liver cancer HepG2 and U87MG glioblastoma cell lines (passage no. 16 and 25) were purchased from NCCS Pune, India and subsequently cultured in Dulbecco's modified eagle medium (DMEM) containing high glucose along with 10% fetal bovine serum (FBS) and a solution of 1% antibiotic and antimycotic. A humidified $CO_2$ incubator containing 5% atmospheric $CO_2$ and a temperature of 37ºC were utilized to ensure optimal conditions for the growth and maintenance of the cell.

**Cell culture treatment:**
A concentration of 2 mg/ml of the extracts was prepared for treating the cells. Serial dilutions of all the stock solutions were then prepared to have different concentrations. All prepared samples were dissolved in the DMEM media containing less than 1 % DMSO.

**MTT cytotoxicity assay:**
To measure cytotoxicity in HepG2 and U87MG cancer cells, the MTT assay was employed. 1 x $10^4$ cells were seeded in each 96-well cell culture plate well and supplemented with freshly prepared drug solutions at 24 hours, 48 hours, and 72 hours, respectively. The control cells were kept without treatment. Following incubation, cell viability was analyzed using 50 µL MTT stock solutions prepared in 0.5 mg/mL PBS and incubated at 37°C for 3 hours. Finally, DMSO was added to each well to terminate the reaction, and optical density was measured at a wavelength of 570 nm using a multimode plate reader (BIOTEK, SYNERGY H1). The cell viability percentage was measured as follows[19].

$$\text{Cell viability \%} = \frac{TreatedOD - BlankOD}{ControlOD - BlankOD} \times 100$$

**Colony forming assay:**
For colony-forming bioassay, 1500 and 2500 cells were seeded in each well of 96 well cell culture plates and kept for incubation for 48 hours by treating with two different concentrations of ethanolic extract. Following incubation, cells were supplemented with fresh media from time to time and cultured for 3 weeks. Later, the colonies were fixed using ice-cold methanol, and further staining was done with 0.1 % crystal violet solution. The cell colonies formed were counted, and comparative analysis was carried out with the number of colonies formed without treatment[20, 21].

**DNA fragmentation Assay:**
Approximately 3 lakhs cells were seeded in culture plates and supplemented with ethanolic extract for 24 hours and 48 hours, respectively. After trypsinizing the cells, DNA was extracted using the conventional phenol-chloroform method. The pellets obtained were dried properly and dissolved in 30µl nuclease-free water. Further, electrophoresis was carried out to observe the isolated DNA[22].

**Live-dead Assay:**
The live-dead assay for both cancer cell lines was performed using confocal microscopy. 2x$10^4$ cells were harvested, treated with ethanolic extract at various concentrations, and incubated for 24 hours. After the incubation period, the wells, which included cells, were rinsed three times with phosphate-buffered saline (PBS). The cells were then stained with Propidium Iodide (PI) and Thiazole orange (T0) and incubated for 15 minutes at 37°C. To remove the excess stain, cells were washed with PBS three times. The samples were then observed under the confocal microscope using dual-channel activation. For PI, the excitation wavelength is 488 nm, and the radiation wavelength is 512–536 nm. For TO, the excitation wavelength is 543 nm, and the radiation wavelength is 537–700 nm[23].

**Fourier Transformed Infrared spectroscopic (FTIR) analysis:**
For FTIR spectroscopic analysis, samples were carefully prepared and subsequently scanned within a 4000-400/cm range.

**GC-MS spectroscopic analysis:**
The GC-MS spectroscopic method was used to determine the compounds in the ethanol extract of *Z. rhetsa* fruit. The GC (Perkin Elmer) apparatus was used to analyze. Utilizing an Elite 1-column capillary, the compounds were separated. The film is made entirely of dimethypolsiloxane; the column is 60 m by 250 µm long. The carrier gas used was helium, having a split ratio of 1:10. Additional requirements include an initial oven temperature of 60° for one minute, a ramp of 7°C per minute to 200°C, a hold of three minutes, and a ramp of 10°C per minute to 300°C, with a stay of five minutes. The ion source temperature was 160°C, while the injector was at 280°C. The mass spectra of the pieces were obtained between 50 and 600 Da. The relative percentage quantity for each component was computed when the average peak area was compared to the overall area. Turbo Mass was the application used to create chromatograms and mass spectra.

**Molecular docking:**
Toxicity analysis, drug ability rule violations, and binding affinity of the compounds obtained in the GC-MS profiling were performed and shortlisted for molecular docking with the Human Epidermal growth factor receptor (EGFR) using Autodock 4.2[24]. The PubChem database of the National Cancer Institute (https://www.ncbi.nlm.nih.gov) provided the structure while the protein data bank (https://www.rcsb.org/) provided the protein's 3D structure (PDB ID: 4LQM)[25].

Then, using Pymol tool, the structure was more refined by getting rid of the co-attached molecules and ions. Further, using Rasmol tool ((http://www.openrasmol.org/) and Ramachandran plot, the 3D structure of the protein was visualized. Procheck (http://www.ebi.ac.uk/thorntonsrv/databases/cgibin/pdbsum/) was used to carry out the statistical analysis. Accelrys Discovery Studio Visualizer (Dassault SystÃ¨mes BIOVIA 2017) and UCSF Chimera version 1.14 were used to investigate the interactions between binding proteins of the docking complexes. In the protein-ligand docking investigations, the molecules (ligands) were maintained flexible, and the protein was kept stiff. The ligands and protein had Gasteiger charges and polar hydrogen added. The molecular docking study's binding energy data explained each ligand's binding interaction and affinity to the target protein. These were expressed as a docking score.

**RESULTS:**

**Extraction and percentage yield of the fruit of *Z.rhetsa*:**

The solvent extraction was conducted by established protocol, utilizing water, methanol, ethanol, chloroform, and ethyl acetate. The percentage yields of *Z. rhetsa* fruit extracts in water, methanol, ethanol, chloroform, and ethyl acetate were 10.2%, 14.5%, 16%, 8.8%, and 5.9%, respectively.

**Phytochemical screening of *Z.rhetsa* fruit:**

The presence of several phytochemicals was assessed in *Z. rhetsa* fruit extracts. Results (Table 1) showed that the *Z. rhetsa* fruit extracts in water, methanol, ethanol, and chloroform included phenols, flavonoids, and alkaloids, whereas the water, methanol, and ethanol extract contained steroids. All four extracts had fixed oils and fats; only the water extract contained amino acids.

**Table 1. Preliminary phytochemical investigation**

| Phytochemicals | Test name | Water | Methanol | Ethanol | Choloroform | Ethyl acetate |
|---|---|---|---|---|---|---|
| Alkaloids | Wagner's test | +ve | +ve | +ve | +ve | -ve |
| | Hager's test | | | | | |
| | Dragondroff's test | | | | | |
| Anthraquinone glycoside | Borntrager's test | -ve | -ve | -ve | -ve | -ve |
| | Modified Borntrager's test | | | | | |
| Saponins | Foam test | -ve | -ve | -ve | -ve | -ve |
| | Bromine water test | | | | | |
| Steroids and Triterpenoids | Salkowaski test | +ve | +ve | +ve | -ve | -ve |
| | Libermann Burchard test | | | | | |
| Phenols | Lead acetate test | +ve | +ve | +ve | +ve | +ve |
| Flavonoids | Shinoda test | +ve | +ve | +ve | +ve | +ve |
| | Alkaline reagent test | | | | | |
| Carbohydrates | Molisch's test | -ve | -ve | -ve | -ve | -ve |
| | Caramelisation | | | | | |
| Tannins | Ferric chloride test | -ve | -ve | -ve | -ve | -ve |
| | Alkaloid test | | | | | |
| | Potessium dichromate test | | | | | |
| Proteins | Biuret test | -ve | -ve | -ve | -ve | -ve |
| Fixed oils and Fats | Sodium hydroxide test | -ve | +ve | +ve | +ve | +ve |
| | Stain test | | | | | |
| | Saponification | | | | | |
| Amino acids | Ninhydrin test | +ve | -ve | -ve | -ve | -ve |

**The total alkaloid, flavonoid and phenolic content of the fruit extracts of *Z rhetsa*:**

**Table 2: Total alkaloid, flavonoids and phenol content of the extracts**

| Extract | Concentration (µg/ml) | Total alkaloid content (mg/g) | Total flavonoid content (mg QE/g) | Total phenolic content (mg GAE/g) |
|---|---|---|---|---|
| Water | 1000 | 34.7 ± 0.98 | 13.55 ± 0.54 | 33.63 ± 0.39 |
| Methanol | 1000 | 59.10 ± 1.73 | 110.39 ± 3.25 | 57.53 ± 0.48 |
| Ethanol | 1000 | 5.59 ±0.55 | 5.15 ± 0.44 | 18.08 ± 0.33 |
| Choloroform | 1000 | 18.57 ± 0.7 | 12.83 ± 0.41 | 12.69 ± 0.33 |
| Ethyl acetate | 1000 | - | 2.66 ± 0.07 | 12.69 ± 0.33 |

For 1000 µg/ml of all the extracts, the methanol extract displayed highest total alkaloid (59.10±1.73), flavonoid (110.39±3.25) and phenolic (57.53±0.48) content in the *Z. rhetsa* fruit extracts. However, since preliminary

screening displayed no detection of alkaloids in ethyl acetate therefore total alkaloids estimation was not performed for ethyl acetate extract of the sample.

**Effect of *Z. rhetsa* fruit extracts on anti-oxidant activity:**

Fruit extracts from *Z. rhetsa* exhibited DPPH radical scavenging antioxidant activity in water, methanol, and ethanol. The $IC_{50}$ values are 428.72 ± 20.96, 333.95 ± 4.73, 378.83 ±2.33 µg/ml respectively. For the standard ascorbic acid, the $IC_{50}$ value was 56.28 ±3.34 µg/ml.

**Effect of fruit extracts of *Z. rhetsa* in *in vitro* cytotoxic activity in HepG2 and U87 MG cells:**

To assess the in-vitro cytotoxic impact of *Z. rhetsa* fruit extracts, HepG2 and U87 MG cells were exposed to the extracts for 24 hours, 48 hours, and 72 hours, respectively. In a dose-dependent manner, every extract exhibited an inhibitory effect against both cell lines (Fig. 1A to 1F). However, out of all the extracts, ethanolic extract remarkably decreased cell viability in concentration and time dependent manner in HepG2 cells at 48 hours and U87MG cells at 72 hours. Table 3 provide the findings for the $IC_{50}$ values in both cell lines. The findings indicate that, in comparison to other extracts, the ethanolic extract of *Z. rhetsa* showed a minimal $IC_{50}$ value of 101.35 ±6.875 µg/ml at 48 hours for HepG2 and 146.99 ±7.7 µg/ml at 72 hours for U87MG cells. Subsequently, the ethanolic extract was selected for further testing.

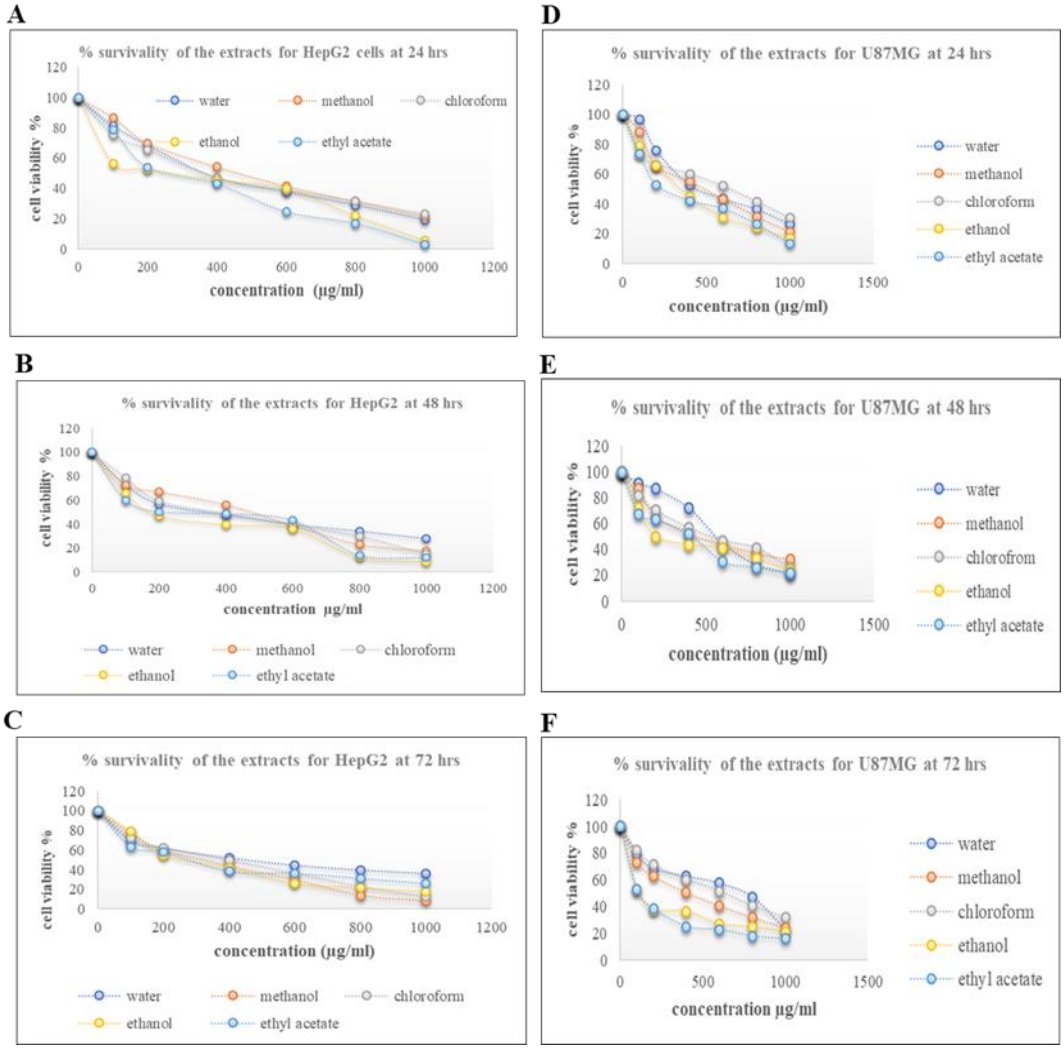


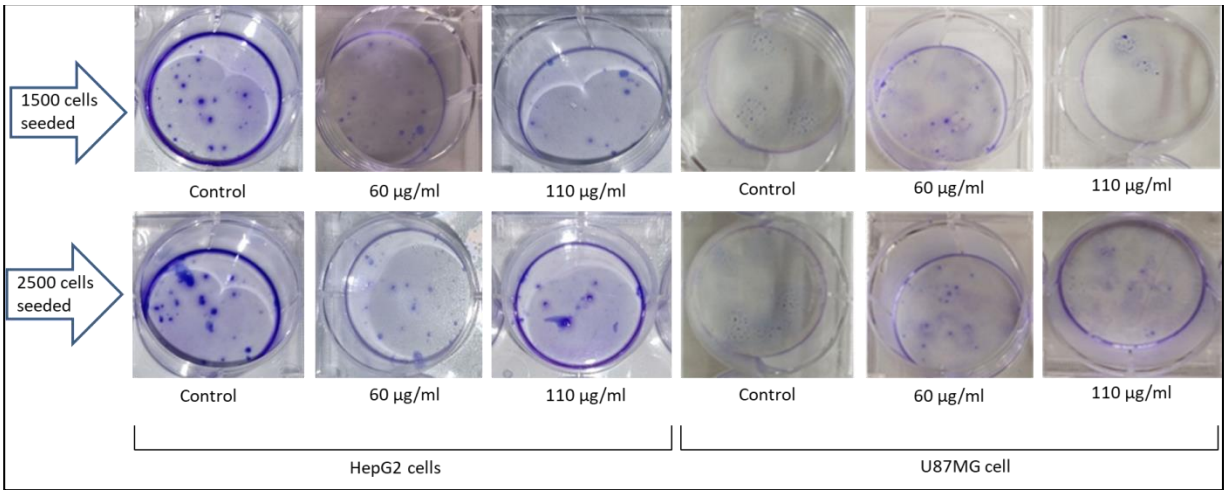

**Figure 1: % survivality graph of the *Z. rhetsa* fruit extracts for (A) HepG2 cells at 24 hours, (B) HepG2 cells at 48 hours, (C) HepG2 cells at 72 hours, (D) U87MG at 24 hours, (E) U87MG at 48 hours, (F) U87MG at 72 hours.**

**Table 3: IC50 value of the extracts for HepG2 and U87MG cell line at different time intervals**

| Cell line | Time Interval | Water | Methanol | Ethanol | Chloroform | Ethyl acetate |
|---|---|---|---|---|---|---|
| | | IC50 (µg/ml) | | | | |
| HepG2 | 24 hours | 483.57± 9.48 | 515.49± 14.15 | 124.92± 4.98 | 480.59± 17.51 | 159.97± 8.83 |
| | 48 hours | 447.52± 10.31 | 471.95± 17.04 | 101.35± 6.88 | 450.35± 8.98 | 149.49± 6.52 |
| | 72 hours | 495.02± 35.84 | 355.53± 7.91 | 180.98± 11.39 | 404.71± 4.99 | 146.98± 6.56 |
| U87MG | 24 hours | 608.69± 18.32 | 537.28± 8.17 | 268.47± 12.66 | 592.45± 11.21 | 211.28± 13.82 |
| | 48 hours | 590.59± 10.41 | 545.33± 24.79 | 201.34 ± 15.74 | 565.26± 13.89 | 194.24± 9.58 |
| | 72 hours | 622.57± 13.27 | 441.2± 20.07 | 146.99± 7.7 | 603.33± 30.2 | 198.22± 11.09 |

**Effect of fruit extracts of *Z.rhetsa* on colony formation in HepG2 and U87MG cells:**

HepG2 and U87MG cells were treated with an ethanolic extract for 24 hours, and their proliferation was measured using colony-forming assay. The results showed that the number of colonies got reduced as the concentration of the extract increased, as shown in Fig. 2.




**Figures 2: Effect of fruit ethanolic extract of *Z. rhetsa* on HepG2 and U87MG cell proliferation for 24 hours at different doses.**

**Effect of fruit extracts of *Z. rhetsa* apoptosis induction in HepG2 and U87MG cells:**

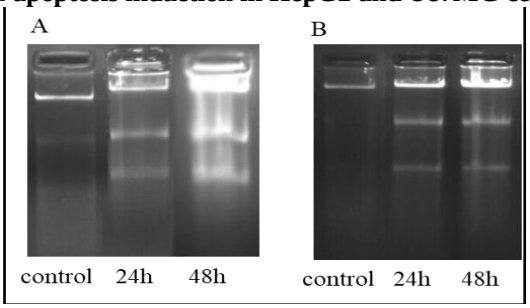



**Figure 3: Effect of fruit extracts of *Z.rhetsa* on induction of apoptosis in (A) HepG2 and (B) U87 MG cells at 24 hours and 48 hours.**

Figures 3 (A) and (B) showed the impact of *Z. rhetsa* ethanolic extract on genomic DNA fragmentation. The genomic DNA of the treated cells showed a laddering pattern in both HepG2 and U87MG cell lines at 24 hours and 48 hours, while the non-treated DNA showed a single band. Therefore, these findings imply that the cells' cellular metabolic activity may have been impacted, contributing to the ethanolic extract's ability to induce apoptosis in cancer cells.

**Effect of fruit extracts of *Z. rhetsa* on induction of apoptosis HepG2 and U87MG cells via Live-dead Assay:**

Comparing the ethanolic extract treatment of 100 and 200 µg/ml with the control, figures 4B and 4C demonstrated that the treatment detached the cells while the control being intact (Fig. 4A). The cell detachment coincides with apoptosis produced by the ethanol extract, as evaluated by two-color fluorescent live-dead labeling, with green indicating viable cells and orange representing dead cells. The detachment increases with the dose, as indicated by the number of deceased cells corresponding to the higher extract concentration.

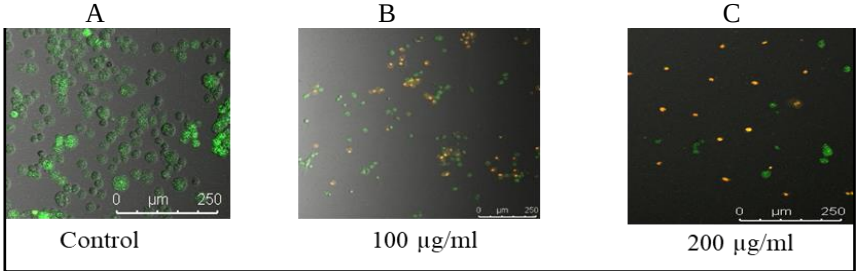



**Figure 4: Effect of fruit extracts of *Z. rhetsa* on induction of apoptosis HepG2 and U87MG cells. (A) Control, (B) 100µg/ml, (C) 200 µg/ml**

**Fourier Transformed Infrared (FTIR) spectroscopy:**
Figure 5 displays the FTIR spectra of the fruit ethanol extract. The 3316 $cm^{-1}$ and 2966 $cm^{-1}$ bands are likely associated with OH groups, aliphatic vCH2, and C-H, which are most likely related to ethanol, respectively[25]. $CH_2$ group's symmetric and asymmetric vibrations are associated with the band 2854 $cm^{-1}$ [26]. The band at 1704 $cm^{-1}$ may be caused by the C=O bonds of the ester groups resulting from fatty acids[27, 28], as well as the C=O stretching of flavonoids[29]. The band at 1625 $cm^{-1}$ may be related to the stretching vibration of C=C groups[28] of phenolic acids, aromatic ring deformations[27], the presence of flavonoids and amino acids resulting in stretching vibration of C=O and C=C, and the asymmetric bending vibration of N–H[29]. The band at 1555 $cm^{-1}$ may be related to the C=C aromatic conjugation of phenols. The band at 1443 $cm^{-1}$ is also associated with phenolic compounds[30], and the band at 1366 cm-1 could result from δ ($CH_2$) twisting and wagging. The band at 1256 $cm^{-1}$ and 31.1049 $cm^{-1}$ may be caused by the polyols' C-O groups vibrating; this may be connected to the presence of primary alcohol and C-O stretch.

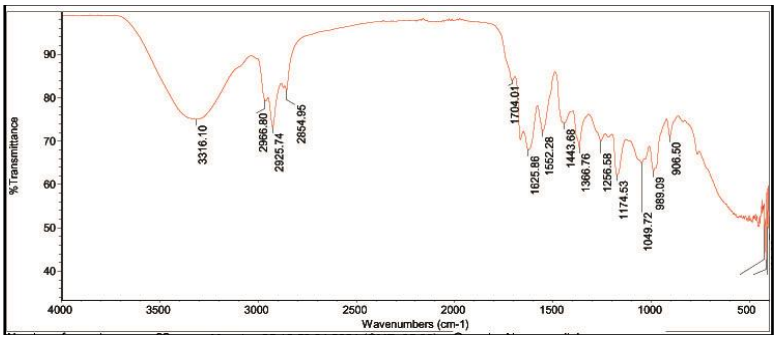



**Figure 5: FTIR spectra of the ethanolic fruit extract of *Z. rhetsa***

**Detection of compounds in ethanolic fruit extract of Z.rhetsa using GC-MS:**
GC-MS analysis revealed a total of 68 phyto-constituents in the ethanolic fruit extract. All the components were compared to those listed in the National Institute Standards and Techniques (NIST 2008) and listed chronologically according to their retention time and peak area percentage (Supplementary Table 1).

**Molecular Docking:**
Based on toxicity analysis, drug ability rule violations, and binding affinity of the compounds, only Nerolidol 1 compound was shortlisted with no toxicity, without violations of rules and low binding energy. The compound Nerolidol 1 with molecular weight 222.37 g/mol and 4.83 XlogP3 displayed 7 bonds which comprise 1 H-bond acceptors and 1 H-bond donor.

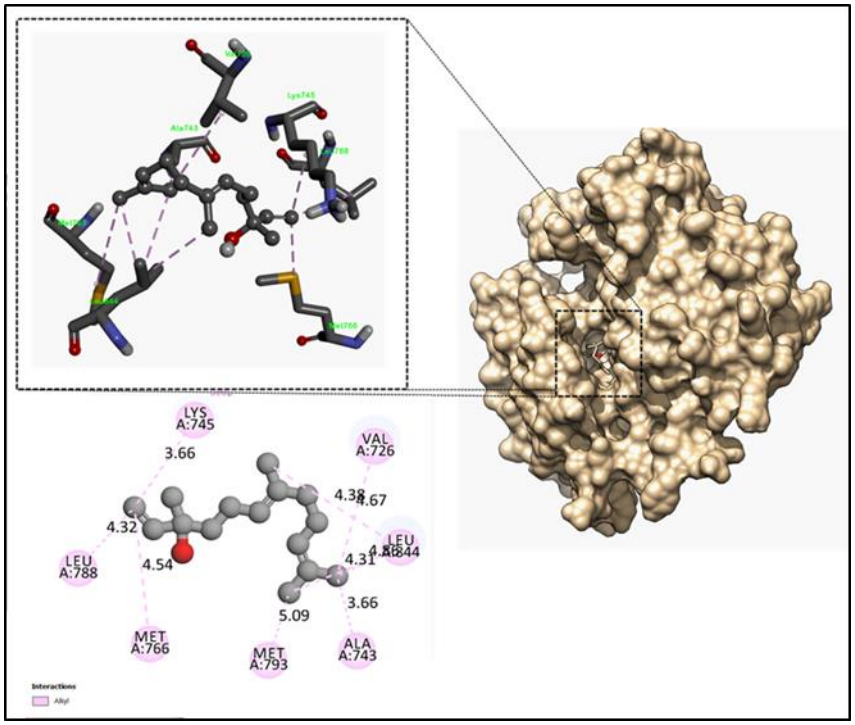



**Fig 6: Nerolidol 1 docking interaction with Human Epidermal growth factor receptor**

The 3D structure and the Ramachandran plot of Nerolidol 1 were generated (Supplementary Fig 1). The 2D interaction showed that Nerolidol 1 maintained the interactions favored by alkyl coordination with Leu788,

Met766, Met793, Ala734, Leu844, Val726, and Lys745 residues of the receptor protein (Fig.6). It was determined that the protein-ligand complex possessed a binding energy of -5.8 kcal/mol. Nerolidol 1 has been revealed to have reduced free energy interaction with EGFR. This suggests that this compound may cause carcinoma cells to activate the apoptotic protein, making it a strong and promising anticancer drug.

**DISCUSSION:**

This study highlights the possibility of scientific evidence of a traditional knowledge-based medicinal plant, *Z. rhetsa*, as a remedy for exerting an anti-cancer affect. Most secondary metabolites, commonly known as natural products, are derived from natural sources, with a higher diversity being found amongst the plants and microbes[31]. These natural products, a rich source of bioactive phyto-constituents, play a crucial role in identifying potential leads for the drug discovery process to treat human maladies[32].

Secondary metabolites such as alkaloids, flavonoids, and isoprenoids are crucial in drug discovery. The discovery of naturally occurring anti-cancer compounds has made it possible to synthesize these substances[33, 34]. Alkaloids, an important secondary metabolite class, serve as a rich repository for major bioactive components. Numerous studies have shown that these alkaloids possess anti-proliferative and anti-metastasis properties in both in vitro and in vivo conditions. Camptothecin and vinblastine, two well-known bioactive compounds, have been extensively promoted as anti-cancer medications[35]. It has been observed that Sanguinarine, another benzophenanthridine alkaloid, possesses antiangiogenic and anti-invasive characteristics, both in vitro and in vivo[36].

Polyphenols represent compounds with advantageous characteristics, including anti-inflammatory, anti-carcinogenic, antiviral, and antioxidant capabilities. A polyphenol-containing extract's bioactivity results from free hydroxyl groups and the functional group attached to the aromatic phenol ring[37-39]. Another research claims that an extract's strong total phenolic content is primarily responsible for its antioxidant qualities[40]. The phenomenon of stress induces a higher generation of reactive oxygen species (ROS) compared to enzymatic antioxidants, culminating in cellular demise[41]. To lessen the harm that free radicals ROS bring to cells, antioxidants must be utilized as scavengers of free radicals. Since rising levels of oxidative stress have been linked to the development of several life-threatening illnesses, antioxidant molecules are essential in everyday living due to their capacity to absorb, neutralize, and repair the damage produced by free radicals[42]. Furthermore, phyto-constituents with strong antioxidant properties may prevent cancer[43]. It has been observed that phenolic compounds, such as gallic acid and its derivatives, have therapeutic properties like anti-oxidant, apoptotic, and pro-oxidant toxicity[44, 45].

Flavonoids are notable secondary metabolites demonstrating antioxidant, anti-inflammatory, anti-carcinogenic, anti-allergic, and gastro protective properties[45-47]. Flavonoids have been shown to exert inhibitory effects on several P450 isozymes, including CYP1A1 and CYP1A2[48, 49], hence exhibiting a protective role against the initiation of cellular harm resulting from the activation of carcinogenic substances. This implies that flavonoids have chemo preventive properties against the development of cancer.

*Z. rhetsa* (Roxb.) DC, which is usually referred to as tirphal, is a member of the Rutaceae family. The species has a vast distribution throughout many regions, including Konkan, Deccan, Mysore, Orissa, Assam, and Meghalaya, as well as the Western and Eastern Ghats of the Indian peninsula. The medicinal herb *Z. rhetsa* has been shown to possess astringent, aromatic, and digestive qualities[50]. The plant has many secondary metabolites, including terpenoids, xanthyletin, sesamin, and alkaloids[51, 52]. According to research, the observed cytotoxic impact of *Z. rhetsa* bark on B16-F10 cells was hypothesized to be attributed to bioactive lignans and alkaloids[13]. Furthermore, it has been shown that the essential oil derived from *Z. rhetsa* has significant potential in inhibiting the proliferation and survival of breast cancer cells, thereby displaying remarkable anti-cancer properties[15].

In our investigation, examination of the diverse *Z. rhetsa* fruit extracts identified secondary metabolites, including phenols, steroids, alkaloids, and flavonoids. On the other hand, fixed oils and fats were discovered in all extracts except the water extract, while amino acids were only found in the water extract. According to quantitative estimates, the methanol extract has the largest concentration of phenols, flavonoids, and alkaloids compared to other extracts. The antioxidant activity was seen in all the extracts except for ethyl acetate.

The inhibitory impact of the extracts was shown to be dose-dependent in both cell lines. However, it was observed that the ethanol extract had a noteworthy inhibitory impact in HepG2 cell lines, particularly after 48 hours. The extract demonstrated an $IC_{50}$ value of 101.35 µg/ml. In contrast, it was shown that the ethanol extract had a noteworthy inhibitory impact on U87MG cell lines after a 72-hour exposure, with an inhibitory concentration ($IC_{50}$) of 146.99 µg/ml. Furthermore, the anti-proliferative effect was validated by the observed decrease in colony formation with increasing concentration of the ethanol extract. The fragmentation of genomic DNA is a crucial aspect of apoptosis[21]. In HepG2 and U87MG cells, it was shown that the ethanol extract of *Z rhetsa* caused DNA fragmentation, which was recognized as a characteristic ladder pattern of apoptotic mode and indicated apoptosis-induced cell death. In a live-dead experiment, propidium iodide and ethidium bromide staining of the cells further demonstrated apoptosis. Furthermore, the FTIR spectra of the fruit extract dissolved in ethanol indicated the existence of functional groups associated with phenols, flavonoids, and fatty acids. This observation aligns with the findings from the first phytochemical screening of the extract.

According to GC-MS analysis, 68 components were found in the ethanolic fruit extract. Among these, a naturally occurring sesquiterpene alcohol called nerolidol has been linked to anticancer properties. It causes G1 phase arrest by down-regulating Akt phosphorylation. Additionally, it may damage DNA and downregulate proteins linked to the cell cycle, which reduces the growth of leiomyoma cells[53].

In their study, Ryabchenko et al. (2008) documented the significant anti-tumor properties of a nerolidol combination consisting of 40.7% cis-nerolidol, 58.3% trans-nerolidol, 0.4% cis-dihydronerolidol, and trans-dihydronerolidol. Furthermore, the vitality of HeLa cells decreased at a concentration range of 1.5-0.7 mM[53]. Again, an additional research investigation documented the utilization of *Zornia brasiliensis* to isolate a refined form of trans-nerolidol. This compound exhibited robust anti-cancer properties against various cancer cells, including human promyelocytic leukaemia (HL-60), human hepatocellular carcinoma (HepG2), murine melanoma B16-F10, and human chronic myelocytic leukemia K562. No cytotoxic activity was seen on non-tumor cells, namely peripheral blood mononuclear cells, despite the presence of though[54].

*In-silico* approaches, such as virtual screening and molecular docking, have made the drug discovery process more predictable[55]. Utilizing molecular docking techniques enables the identification of the precise binding of a ligand within the site of activity of the target or receptor protein. As a result, the binding pattern and proper location of the lead molecule within its receptor may be more reliably determined. The protein molecule under analysis in our study was EGFR, also known as epidermal growth factor receptor, which has been often seen to be overexpressed in instances of hepatocellular carcinoma (HCC). From the GC-MS profiling, only Nerolidol 1 was chosen based on the compounds' toxicity analysis, drug ability rule violations, and binding affinities. It was subsequently docked to Human EGFR. The researchers observed that the protein-ligand combination exhibited a binding energy of -5.8 kcal/mol. Determining the degree of interaction between a ligand and a protein within a complex relies upon the binding energy, which concurrently provides crucial insights into the binding affinity of the protein and its potential biological impact[56]. A lower binding energy corresponds to a higher potential impact. Given that our research revealed nerolidol1 to have a reduced free energy interaction with EGFR, this compound may cause carcinoma cells to activate the apoptotic protein, making it a strong and promising anticancer drug.

In summary, our study presents novel findings indicating that the fruits of Z. rhetsa sourced from North East India can inhibit human cancer cell lines in a dose-dependent way. Notably, the ethanol extract exhibited the highest potential among the other extracts tested. Nevertheless, the observed variation in bioactivity within the crude extracts indicates that the phyto-constituents, such as alkaloids, flavonoids, or phenols, present in these extracts may influence distinct mechanisms of apoptosis. Therefore, the plant has potential as a viable candidate for discovering and developing novel lead molecules with anticancer properties. Nevertheless, it is highly advised to conduct further procedures such as isolation, purification, characterization, and standardization of the bioactive components and investigate their mechanism of action to authenticate their efficacy.

**CONFLICT OF INTEREST:**

The study is devoid of any conflicts of interest.

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