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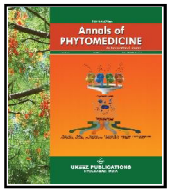

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In vitro assessment of cytotoxic effects of guggul in L929 mouse skin fibroblast cells

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Abstract

Cancer is one of the major causes of death after cardiovascular diseases worldwide. Guggul is gum oleo-resin exudates from *Commiphora wightii* (Arnott.) Bhandari with immense medicinal properties. Guggulsterone (GS) is one of the main constituent of guggul. During the present study three samples of gum oleo-resin of *C. wightii* were evaluated for their cytotoxic activity against L929 mouse skin fibroblast cell lines. In our study, the cytotoxic responses of guggul was determined by the 3-(4,5-dimethylthiazol2yl)-2, 5-biphenyl tetrazolium bromide (MTT), neutral red uptake (NRU) assays and lactate dehydrogenase (LDH) release assay. The initial analysis indicated that guggul samples tested has exhibited cytotoxicity. The outcome has opened a new window of possibility for the potential use of guggul against L929 cells.

1. Introduction

After cardiovascular illnesses, cancer is a serious cause of death globally (GBD, 2015). Cancer in the human body can result from abnormal cell growth of any variety (Cooper, 2000). According to the World Cancer Research Fund, there were around 18 million new instances of cancer worldwide in 2018. Since the advent of precision medicine, the survival rate of cancer patients has increased in developed nations. In underdeveloped and developing nations, prolonged treatment and the cost of treatment are important socioeconomic obstacles (Bhatt *et al.*, 2019; Desai *et al.*, 2021). Numerous studies are being conducted to find ways to treat cancer more affordably and with fewer casualties using medicinal herbs (Purushothaman and Kuttan, 2019; Mathew *et al.*, 2019).

One of the most significant ways of treating illnesses in India is Ayurveda (Chanchal *et al.*, 2018). Numerous plants, including trees, shrubs and herbs, are recognised to have medicinal effects. Guggul is a therapeutically important oleo-resin obtained from a shrub, *C. wightii*. This important medicinal plant species generally grows in semi-arid and desert climates (Thomas *et al.*, 2011).

Guggulsterone (GS) is one of the significant ingredients of guggul gum oleo-resin and *C. wightii* is one of the significant pharmacological plants initially mentioned in the Atharva Veda (2000 B.C). According to the *Sushrut Samhita*, guggul purportedly treats abrupt paralytic seizures, edoema, leucoderma, internal tumours, malignant sores and ulcers, internal tumours, obesity and liver failure (Shishodia *et al.*, 2007).

An important sterol derived from guggul is GS (4, 17 [20]-pregnadie 3, 16-dione). The farnesoid X receptor, which is necessary for healthy lipid and glucose metabolism, is antagonistic to GS (Cui *et al.*, 2003). Additionally, *in vitro* studies using PC-3, DU145 and LNCaP human prostate cancer cells revealed that the trans-(z)-isomer of GS caused apoptosis and decreased cell proliferation (Singh *et al.*, 2005; Singh *et al.*, 2007). Other cell types, such as human lung, acute myeloid leukaemia and breast cancer, have also demonstrated GS's anticancer capabilities (Samudio *et al.*, 2005).

Poor handling and storage of guggul is known to cause degradation in GS content (Thomas *et al.*, 2020). Due to the severe shortage and increasing demand, adulteration is another issue to be concerned about. In light of this, the present investigation was created to assess the anticancerous activity of pure guggul gum with various storage times.

2. Materials and Methods

Three samples of gum oleo-resin of *C. wightii* were evaluated for their cytotoxic activity. Three age group of stored guggul gums were termed as sample I, II and III. Fresh guggul gum (Figure 1) were collected immediately after tapping and brought to the laboratory for its storage at room temperature. Guggul gum was stored (Figure 2) for different periods 4 months (fresh sample I), 12 months (sample II) and 24 months (sample III). Baked earthen clay pots of capacity 500 ml with a wall thickness of 2.5 mm was used for storage. All the pots with guggul gum were covered with earthen lid. The pots were

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made by local potter. The cytotoxicity of the three guggul samples against L929 mouse skin fibroblast cell lines was carried out in the CSIR-Indian Institute of Toxicological Research, Lucknow, India following the guidelines of Indian Pharmacopoeia. To ascertain whether a substance contains appreciable levels of biologically hazardous extractables, cytotoxicity testing is a quick, standardised, sensitive and affordable method. The high sensitivity of the tests is a result of the observation of the effect of the test item on the isolated, purified population of cells in culture medium without the protective systems in contrast to the entire body system.

Figure 1: Fresh guggul gum oleo-resin.

Figure 2: Stored guggul gum oleo-resin.

2.1 Cell lines

An authorised cell line per ISO-10993-5 for cytotoxicity investigations is the L929 mouse skin fibroblast. The L929 cell line was first purchased from the National Centre for Cell Sciences in Pune, India, and was afterwards kept at the CSIR-Indian Institute of Toxicology Research in Lucknow, India, in accordance with the established protocol.

2.2 Culture initiation

Cells in frozen vials (L929) were taken out from the liquid nitrogen containing cylinder and transferred to a water bath that had been preheated to 37°C. After that, the cells were put into sterile centrifuge tubes with complete medium (MEM + 10% FBS + antibiotic-Antimycotic solution-100x (1.0 ml/100 ml of medium) + sodium bicarbonate (0.2%), as well as the addition of cell-specific supplements were taken in vials. The vials were then centrifuged at 600 rpm for 10 min at room temperature. After centrifugation, the produced supernatant was thrown away. In 5 ml of complete medium, the last remaining loosely bound pellet was suspended. After that, the right amount of cells were seeded in T-75 tissue culture flasks and cultured at 37°C in a CO_2 incubator (5% CO_2 - 95% air at high humidity). Once sufficient growth was seen, the medium was replaced after a gap of 2-3 days and then every 24 h at first. Prior to using the cells in the research, the vitality of the cells was tested using the trypan blue dye exclusion assay. For the investigation, only batches containing more than 95% viable cells were employed.

2.3 Preparation of test material

The solubility of each of the three guggul gum samples was evaluated in terms of culture media, water, DMSO, DFM and methanol. In comparison to DMSO, all three samples were shown to be extremely soluble in culture media, water and methanol (Culture grade from Sigma Chemicals, St. Louis, MO, USA). In order to generate a stock solution that had a much higher concentration than the experimental concentrations, DMSO was utilised as the medium. To make the stock solution more transparent, the stock solution was subjected to three rounds of sonication, each lasting 20 KHz for 10 seconds. The pH was then adjusted to 7.4 and dilution of the stock solution was taken place. Different concentrations, *i.e.*, 10 mg/ml to 0.0001mg/ml were prepared after dilution. In order to maintain the concentration of DMSO in the experimental solution below 0.01%, the stock concentration was created. Therefore, there was no need for a second vehicle control group. Prior to use, a membrane filter with a bore size of 0.22 M was used to filter all test material concentrations.

2.4 Cytotoxicity assessment (MTT Assay)

The MTT assay shows that live cells have the ability to use mitochondrial dehydrogenase to change the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium salt into a purple MTT. This was determined spectrophotometrically at 530 nm. The test gives a sign of the health and activity of the mitochondria, which is translated into a percentage of cell viability. The assay was carried out using the technique recommended by Siddiqui *et al.* (2008).

In order to ensure appropriate cell attachment during the experiment, cells ($1x10^{-4}$) were planted in 96-well tissue culture plates and cultured for 24 h at 37°C in a CO_2 incubator. The medium's aspiration came after it. After being rinsed with PBS (pH 7.4), the cells were then cultured in media with varying test material concentrations (10 mg/ml to 0.0001 mg/ml). To act as a basal control, a parallel set of cells was also grown under the same experimental circumstances without contact with the test substance. To act as a positive control, a group of cells that had been treated to $MnCl_2$ 10-3M was also run concurrently. At 24, 48, 72 and 96 h, MTT assays were performed. To each well containing 100 ml of cell suspension, tetrazolium salt (10 µl/well) was added 4 h before the end of each incubation period. Further, 200 µl of DMSO was added to each well and the mixture was homogenised by pipetting. The color was read at 530 nm after 10 min, using a multiwell reader (Bio-Tek, USA). The percentage of cell viability in the exposed group was calculated using the results obtained from the unexposed control group, which were taken to be 100% accurate.

2.5 Neutral red uptake (NRU assay)

The assay was performed in accordance with the previously reported procedure (Siddiqui *et al*., 2008). The cells were subjected to test substances at varying doses in an experimental setting identical to the MTT assay. The medium was aspirated, 100 µl of NRU salt (50 M/ml in medium) was added to each well plate. The plate was then incubated for 3 h. After carefully removing the reaction mixture, the plates were washed with washing solution (100 µl/well). Further, 200 µl of a combination containing 1% acetic acid and 50% ethanol was added after the washing solution removed. The plates were placed on a rocker shaker for 10 min at room temperature. Further, the plates were examined at 540 nm using a multi-well microplate reader (Bio-Tek, USA). A positive control (a group of cells that had been treated to $MnCl_2$) was also put through the same procedure. To determine the percentage of cell viability in the exposed group, the results obtained from the unexposed control group, which were taken to be 100%, were used. The generated information was compared with the control group,s cell viability percentage.

2.6 Lactate dehydrogenase (LDH) release assay

The LDH release assay is a technique for assessing the reliability of the membrane in relation to the volume of LDH released into the medium. For *in vitro* cytotoxicity assessment, a ready-made commercial LDH assay kit was used (TOX-7, Sigma St. Louis, MO., USA). The assay is based on LDH's impact on NAD to reduce it. The resulting reduced NAD (NADH+) was used to convert a tetrazolium dye in a stochiometric assay. The coloured chemical thus produced was measured at wavelengths of 490 and 690 nm using a multiwell plate reader. Similar experimental procedures to those used for MTT and NRU experiments were used to expose the cells to various test item doses. Following the conclusion of the corresponding exposure times, the cells underwent comparable processing to the MTT assay for the LDH release assay. The culture plates were taken out of the CO_2 incubator and centrifuged at 250 × g for 4 min. After that, the supernatant from each well was transferred to a fresh 96-well flat bottom culture plate. The enzymatic analysis was done following the manufacturer's instruction. Cell viability was compared between the exposed and unexposed groups.

3. Results

All three of the guggul samples showed cytotoxicity at dosages of 10 mg/ml or higher *in vitro* for up to 96 h. Figure 3 summarises the key findings of the results from the cytotoxicity tests of the guggul gum samples (I, II, and III) performed in L929, a mouse fibroblast cell line.

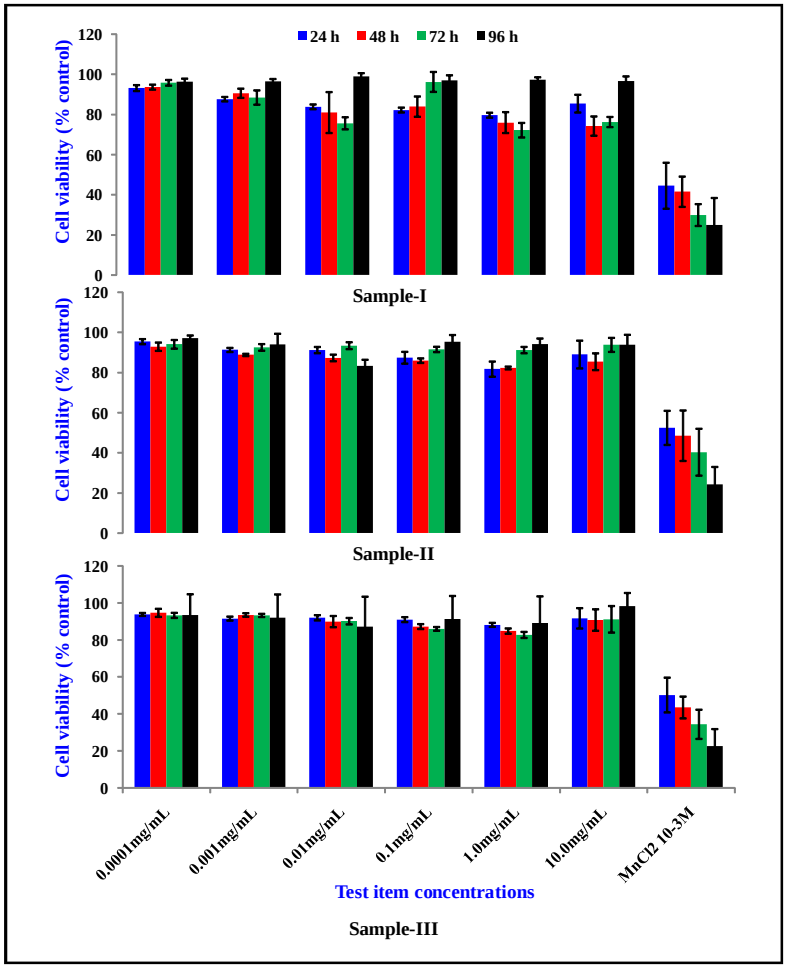

Figure 3: Cytotoxicity analysis of guggul gum (Samples I, II and III) MTT assy in L929 cells.

In response to exposure to various concentrations of all three samples of guggul gum (I, II, and III) at any time point, *i.e.*, between 24-96 hours, the MTT assay revealed a modest decline in the per cent cell viability. Cells treated for a longer time at a concentration of 10 mg/ml exhibited a higher level of cytotoxicity. Nevertheless, the effect was neither physiologically or statistically significant. In general, except few places at higher concentrations the mean ± SD values has no significant deviation from different replicates of experimental groups.

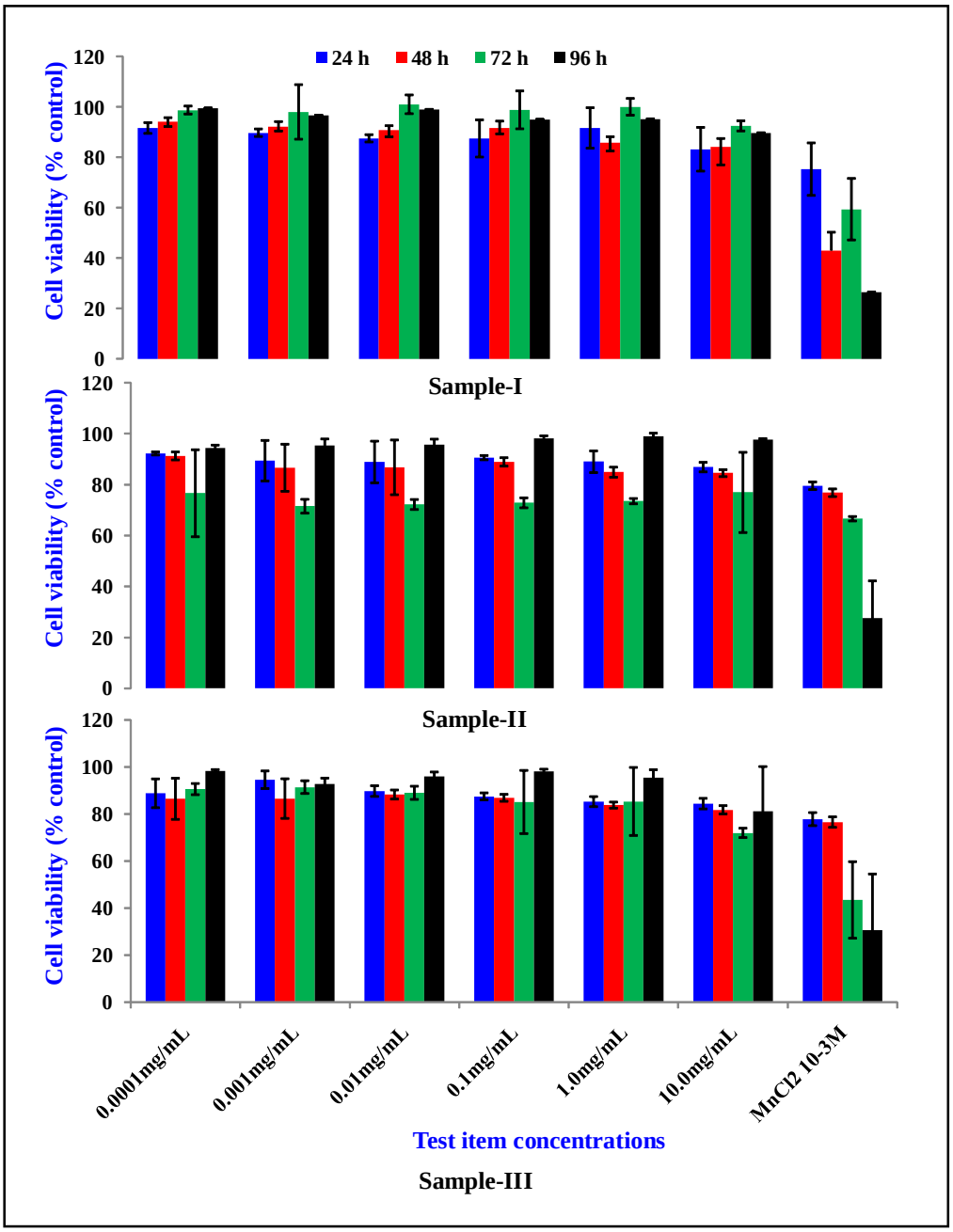

Figure 4: Cytotoxicity analysis of guggul gum (Samples I, II and III) by NRU assay in L929 cells.

The guggul extracts suppressed cell proliferation in L929 mouse skin fibroblast cells in a dose-dependent manner, according the findings of this study. The three guggul samples were tested using the NRU assay against L929 cell lines for 24-96 h of exposure (Figure 4), but no cytotoxicity was detected.

Following exposure to guggul gum samples at varying concentrations (I, II, and III), nominal cytotoxicity was seen in the L929 cells using the LDH assay (Figure 5). Comparing exposed control groups to unexposed groups, the rise in LDH release was noticeably reduced. In fresh guggul exposed for 48 and 96 h and 24 month guggul exposed for 48 h, a minor cytostatic reaction was seen.

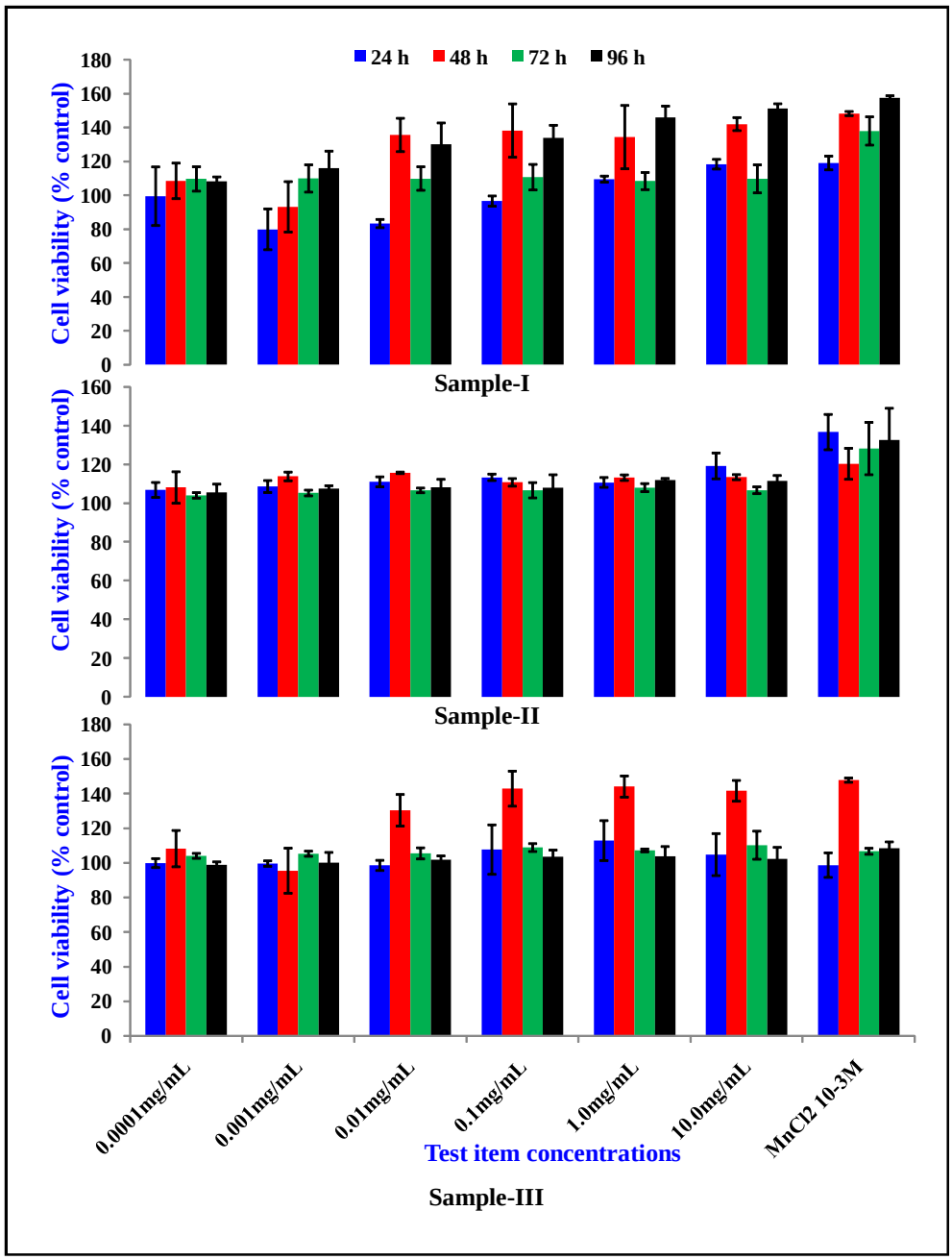

Figure 5: Cytotoxicity analysis of guggual gum (Samples I, II and III) by LDH release assay in L929 cells.

4. Discussion

Utilization of medicinal plants in the treatment of cancer is being followed for many years. Various plants with such therapeutic values and anticancer activities are documented in the literature (Khazir *et al.*, 2014; Iqbal *et al.*, 2017). Among different approaches for the determination of anticancerous activity of plant extracts, MTT, NRU and LDH release assay were applied due to their high efficiency during the current investigation. The MTT assay is generally used to determine cellular metabolic activity as a sign of cell viability, production and cytotoxicity. On the basis of the results of MTT assay increased concentration of guggul extract in combination with increased exposure time resulted in decreasing the number of live cells. Similarly, the *Astragalus elongatus* produced lactate dehyrogenase enzyme activities on human cancer cells in accordance with the findings of the present investigation (Sekeroglu *et al.*, 2019). A typical technique for determining cellular proliferation is the MTT test (Freimoser *et al.*, 1999). This test indicates the presence of functional NADH and NADPH-dependent redox enzyme systems both inside and outside of the mitochondria (Jo *et al.*, 2015). The neutral red uptake assay is a widely utilised cytotoxicity test with a variety of applications that deals with the quantitative evaluation of cell viability (Repetto *et al.*, 2008).

The non-specific rise in LDH release could not be attributed to exposure because it was neither concentration nor time dependent. Because injured cells are totally fragmented during the course of extended incubation with chemicals, LDH is a trustworthy and accurate approach to assess cytotoxicity (Parhamifar *et al.*, 2013). Numerous studies have examined how medicinal plant extracts cause cytotoxicity in cancer cells (Sivalokanathan *et al.*, 2006). Therefore, the cytotoxic quality of the plant extract, which supports its anticancer action, may be the cause of the LDH leakage in the examined cell lines.

Effective anticancer drugs have primarily been derived from plants (Safarzadeh *et al.*, 2014). Extensive ranges of natural compound possess significant cytotoxic as well as chemo-preventive activity which act through apoptosis. Similar properties can also be found in plant extracts used in conventional therapy (Spencer and Jacob, 1999). The susceptibility of diseased cells to the death is reflected in our results, which are in good agreement with earlier findings that plant extracts can cause cytotoxicity in human breast cancer T47D cells (Abdolmohammadi *et al.*, 2008). Other research revealed that specific plant components also suppress the growth of human uterus cancer (HeLa), murine melanoma (B16F10) and human gastric adenocarcinoma (MK-1) cells (Fujika *et al.*, 1999). Various researchers have noted the growth-inhibitory effects of other extracts on malignant cells using different human cancer cell lines (Li *et al.*, 1995; Kim *et al.*, 2002). There has also been evidence of the cytotoxicity of plant extracts, suggesting that the cytotoxicity may be caused by the presence of active components in plants (Samarakoon *et al.*, 2010). Numerous studies have also suggested that the presence of polysaccharides, flavonoids, coumarins, monoterpene glycosides, and alkaloids in various plant extracts may be the cause of these actions on malignant cells (Chanda and Nagani, 2013).

Recently, Ulrich *et al.* (2022) have demonstrated the effectiveness of *Commiphora* against the malignant melanoma cell lines RPMI-7951 and SK-MEL-28 as well as the epidermoid carcinoma cell line A431. They discovered that combining the effects of the two isomers of guggulsterones E and Z is critical in the treatment of cancer. They also came to the conclusion that the anticancerous properties of guggul are due to the existence of additional cytotoxic chemicals known as guggulsterols in addition to guggulsterone (Patil *et al.*, 1972). But, these important chemicals need to be the subject of biological and pharmacological studies. The results of the present study also highlight the need for additional, in-depth research on phytochemicals in order to locate additional, novel bioactive compounds in *C. wightii* that have cytotoxic effects. These discoveries will aid in the creation of new anticancer medications.

4. Conclusion

An alternate supply to the expensive anticancer chemotherapeutic chemicals may be found by screening herbal medicines for mutagenicity. Some medicinal plants have gained popularity as non-toxic and inexpensive cancer treatments due to their low toxicity. In this investigation, NRU, LDH and MTT assays were used to measure the cytotoxic effects of guggul. Although, there was no discernible difference, the preliminary study suggested that the tested guggul samples had demonstrated cytotoxicity. This resulted from a lower dose (10 mg/ml concentration assessed) compared to the manual advised dose of 1000 mg for serious conditions (Szapary *et al.*, 2003). The outcome has, however, created a fresh opportunity for guggul's possible usage against L929 cells.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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