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Harnessing Plant-Based Silver Nanoparticles: Evaluating the Anticancer Potential of Gishta Extracts via Enhanced Caspase-3 Activity

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Integration of plant-based silver nanoparticles as therapeutic agents opens up new horizons for combatting microbial infections and offering targeted therapies for cancer treatment. Nanotechnology is a congenial and proved technology for nanoparticle synthesis over conventional wet chemical techniques as it was proved ecofriendly and feasible over the latter. The study aims at evaluating the anticancer properties, caspase -3 activity of Gishta mediated Ag-NPs extracts in vitro as a potential candidate for pro-apoptotic active ingredients. Organic solvent extracts (chloroform and methanol) of Gishta were evaluated for cytotoxicity effect using the Ames test (MTT Assay) and caspase-3 enzyme activity studies. All the tested fractions exhibited powerful cytotoxicity (95% Cell death), elevated caspase-3 activity. This research concludes that the Gishta mediated Ag-NPs has a higher value of bioefficacy and suggest that it increases caspase-3 activity and fights effectively

against cancer cells.
Keywords: Anticancer, Gishta, MTT Assay and Caspase.

1. Introduction

The utilization of silver nanoparticles (AgNPs) prepared from phyto extracts is a promising strategy for combating microbial infections and addressing the challenges associated with cancer treatment. The integration of nanotechnology with the therapeutic properties of plants presents a unique opportunity to develop effective and targeted approaches for disease control. In this context, silver nanoparticles derived from plant sources have shown great potential due to their inherent anticancer properties (Chhangte Vanlalveni et al., 2021; Yousaf A et al., 2022; Jain, N et al., 2021).

Silver nanoparticles derived from plants have gained attention in cancer treatment research due to their potential advantages like selective toxicity, good penetration into tumor tissues, enhanced drug delivery, synergistic effects, anti-angiogenic (crucial process in cancer progression) properties, antibacterial and antifungal properties (secondary infections can complicates the therapy process and weaken immune patients) (Gomes HIO et al., 2021; Yahaya Gavamukulya et al., 2017; Yao Yihan et al., 2020). Various properties of AgNPs make them promising agents for cancer treatment. Their tiny size and broad surface area would enhance their high cellular uptake, promoting efficient supply of therapeutic agents to cancer cells. Furthermore, silver nanoparticles possess inherent cytotoxic effects against cancer cells, triggering apoptosis (programmed cell death) and inhibiting tumor growth (Gomes HIO et al.,2021; Xu L et al., 2020; Hailong Tian et al., 2022).

Ag substances are widely being used as food supplements, garments, wrapping, and plastics (Panaek, A., et al., 2006 and Wang, Q.F., et al., 2006). Hence, different silver substances are formulated for fabrication of silver nanoparticles (Lu, Y., etal., 2007; Willner, I., et al., 2006; Nadagouda, N., et al., 2007; Chen, M., et al., 2006 and Xiong Wen Lou, et al., 2006). Of widely used routes of silver nanoparticles preparation, toxic chemicals like NaBH4, citrate or ascorbate (Chen, M., et al., 2006 ; Xiong Wen Lou, et al., 2006 and Ping-Lin Kuo & Wei-Fu Chen., 2003) are used as reducing agents. These toxic reducing agents are related to biological hazards. So developing an organic amalgamation of silver nanoparticles is very desired to subside this problem. Moreover, the use of plant-based silver nanoparticles offers several advantages over conventional treatment approaches. Plants are readily available, cost-effective and offer a diverse scope of prominent compounds to prepare nanoparticles. Furthermore, the eco-friendly nature of the green synthesis method minimizes the environmental impact associated with traditional chemical synthesis processes(Habeeb Rahuman HB et al., 2022; Mustapha T et al., 2022; Rosman, N. S. R et al., 2021).

Plant based nanoparticles preparation has been proved simple and eco-friendly hence started gaining importance these days. Gold nanoparticles of Alfalfa (Gardea-Torresdey, J.L., et al., 2003; Gardea-Torresdey, J.L., et al., 2002), Aloe vera (Chandran, S.P., et al., 2006), Cinnamomum camphora (Huang, J., et al., 2007), Azadirachta indica (Shankar, S.S., et al., 2003), Emblica officianalis (Ankamwar, B., et al., 2005), Cymbopogon citratus (Shankar, S.S., et al., 2004) and Tamarindus indica (Ankamwar, B., et al., 2005) are recorded, but still the potentiality of nanoparticles of many plants have to be explored. Plant extracts may contain bioactive compounds with potential pharmaceutical applications. Caspase activity assays can be used as part of a screening process to identify plant-derived compounds that could have pro-apoptotic effects, making them potential candidates for anticancer or antimicrobial drug development (Chaudhry Gul-e-Saba et al., 2022).

Graviola, a short, erect and evergreen tree species grows about 5–6 m long, leaves are shiny and dark green. It produces large, green, heart shaped fruits having white fleshy edible pulp. The fruit measures about 15–20 cm in diameter. The different parts of the plant have been utilized conventionally for treating various ailments (Edwards, S., et al., 2000). Graviola is usually present in the rain forests of different regions of the Earth. The other names of the plant are thorny custard apple, cherimoya and Brazilian pawpaw. The fruit is also called differently in various languages like soursop (regions of America), and cachiman (épineux), shul-ram-fal, hanuman fal, and mullaatha (India), Harar and yebere lib (Ethiopia) means heart of cow (Blackherbals, 2019).

As traditional folkloric medicine seeds and leaves of Gishta are very effective in treating cancer, various parasitic infections etc. Seeds are known for their emetic function, seed oil kills lice, floral parts are antispasmodic in nature, fruit pulp has been used as diuretic and to treat some other ailments. The raw fruit has astringent properties, cures intestinal problems, whereas ripened fruits are used as antiscorbutic and anthelmintic. The beverage is used as medical supplement to treat gaustric problems. The bark is effective in curing diarrhoea and dysentery. The medicinal properties of the species are attributred to bio-active compounds acetogenins (Ruppercht, J.K., et al., 1982). To our knowledge investigation of Gishta for phtochemical composition, antibacterial, antifungal, anticancer, caspase-3 activity of silver nanoparticles has not been done especially on this plant species (Saripalli, Harikrishna & Dixit, Prasanna., 2016).

This study explored on synthesis of AgNPs of Gishta seeds and studying their anticancer activities and caspase-3 enzyme activity using different biological and bioassay methods. Bioactive ingredients in Gishta have a role in inducing toxicity of cell proliferation enzymes, one of which is caspase-3.

2. Materials and Methodology:

2.1 Ghista fruits collection

Fruits of Ghista (Fig. 1a), collected from the Jimma (7°40'26.01"N, 36°50'8.85"E) Oromo region, Ethiopia, in January 2016 after confirmation and authentification deposited voucher specimen number: AAU/CBS/G/2014-01 in College of Biological Sciences, Addis Ababa University, and supplied by Ato Behailu Etana Disasa of Natural Resource Management, College of Agriculture and Veterinary Medicine, Jimma University, Jimma, Ethiopia.

2.2 Extract Preparation

Seeds of Gishta were dried in the shade at 28 ±30C temperature under aseptic conditions. Then the dried seed materials were powered and subjected to standardized Soxhlet procedure using chloroform and methanol to extract the sample (Harikrishna Ramaprasad Saripalli., 2004). Thus prepared sample was preserved at 40C for further research.

2.2.1 Chemicals and Reagents

All chemicals and reagents were procured from certifed suppliers and were of the highest analytical standard.

2.3 Making of Silver Nitrate solution

A quantity of 0.1699g of AgNO3 particles dissolved in 1liter purified water to prepare 1mM solution and it was preserved in dark coloured glass jar for controlling the silver auto-oxidation.

2.4 Synthesis of Ag-NPs

1mM AgNO3 was taken in the flasks containing chloroform and methanol plant extract(s) separately and brought the volume of the solution to 200ml. The mixtures were subjected to the centrifugation at 18,000 rpm for 25min and later exposed to the sand bath for 10 min at 60oc. Each solvent alone without seed extract served as a control. The process of incubation was continued till the solution turned to dark colour. Dried plant extract was subjected to flash evaporator for half- an-hour to get the powered Ag-NPs. The leftover material was treated as the absolute extract powder and different concentrations of the extract powder were prepared by dissolving it in the corresponding organic solvent (Harikrishna Ramaprasad Saripalli., 2007).

2.5 UV-VIS Spectral analysis of Ag-NPs

The synthesis of Gishta mediated Ag-NPs were further confrmed by ultraviolet–visible spectroscopy (UV/VIS). A small quantity of the sample dissolved in the distilled water, suspended for 5 hours and subjected to spectral analysis(Shah M et al., 2015).

2.6 Scanning Electron Microscope (SEM) analysis of Ag-NPs

Studied physical characters of AgNPs using standard procedure and Scanning Electron Microscope (SEM). The re-dispersed nanoparticles were dried in oven to obtain a powdered form and 10mg of the sample was re-dispersed in ethanol. The sample was made into thin layers on a carbon supported copper grid and dried using mercury-vapor lamp.

2.7 In-Vitro Assay of Cytotoxic Activity

The Cytotoxicity of test samples (Gishta mediated Ag-NPs extracts) on HepG2 (human hepatoblastoma cell lines) was determined by the MTT assay (Mosmann et al.,1983; La Fontaine S et al., 1998; Southon A et al., 2004).

2.7.1 Preparation of Media and Reagents

Dulbecco's modified Eagle's medium (DMEM) Prepared using the ingredients like 4.5g/l glucose, 0.110/l sodium pyruvate and L-glutamate and Fetal calf serum (FCS). The medium was further filtered and sterilized with gamma-irradiated at 25-28 kGy to sustain the quality and stored in 50 ml sterile flasks at -20 ºC until use.

0.25% (w/v) trypsin in Ca++ and Mg++ free phosphate buffered saline (PBS).

0.1% (w/v) EDTA in Ca++ and Mg++ free PBS

MTT reagent - 3-[5, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide chemical was prepared by dissolving 5 mg MTT in 1 ml PBS (pH 7.5). The solution was subjected to filter sterilization and preserved at low temperature.

2.7.2 Culturing of Cell

A human hepatoblastoma cell line (ATCC) maintained in tissue culture flasks and incubated at 37°C and 5% CO2 in a humidified incubator. 80% confluent cells were trypsinized.

2.7.3 Treatment of Cell

200µl cell suspension (3x104 cells/ml) was taken into each well of 96-well plate. To each well, 2µl of each of the organic solvent AgNPs extract of Gishta was added in triplicate. Three untreated cultures in wells with only carrier medium were taken as control. Finally the filled, 96 well plate was incubated at 37ºC in humid chamber for 24h.

2.7.4 MTT assay

MTT assay (Figure 4) (Hanelt, M., et al., 1994) was applied to measure the cytotoxicity. For this 20µl MTT reagent was taken in each well after 24 hours of cell incubation and reincubated at 37ºC in a humid chamber about 3 hours. Formazan crystals formed during the incubation were dissolved in 100µl DMSO and suspended the solution for 30min and the absorbance was noted down at 578nm (with a reference wavelength of 630nm) with the help of ELISA reader and the cell viability was calculated in percentage (Reddy, L., 2005). Measurements were performed and the concentration required for a 50% inhibition of viability (IC50) was determined graphically. The absorbance at 578 nm was measured with a UV- Spectrophotometer using wells without sample containing cells as blanks. The effect of the samples on the proliferation of HepG2 was expressed as the % cell viability, using the following:

$$\text{\% of Cell viability} = \frac{\text{No. of cells treated}}{\text{No. of cells untreated}} \times 100$$

% of cell death = 100% - percentage of cell viability

Figure 1 a: Gishta (Annona spp.) fruits of Ethiopia

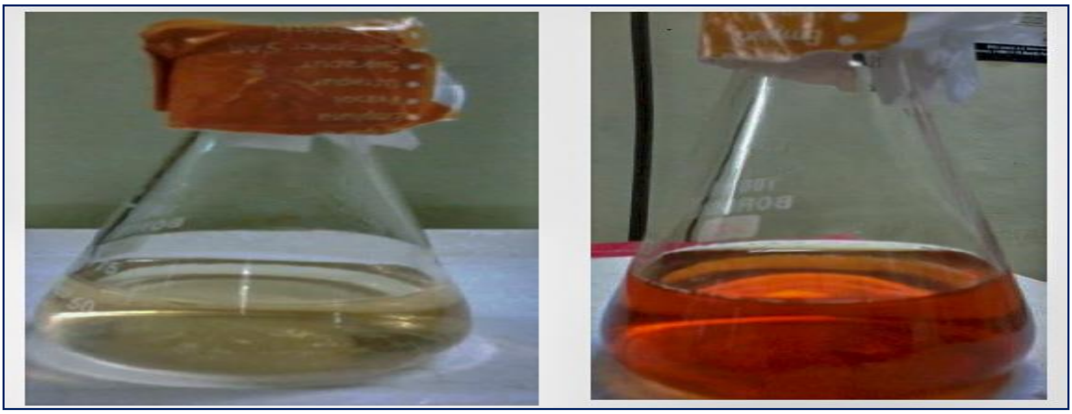
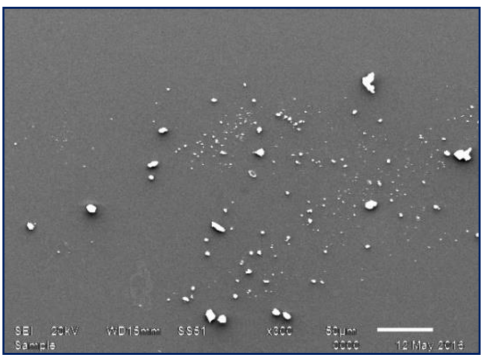


Figure 1 b: Change in the colour assures AgNPs synthesis



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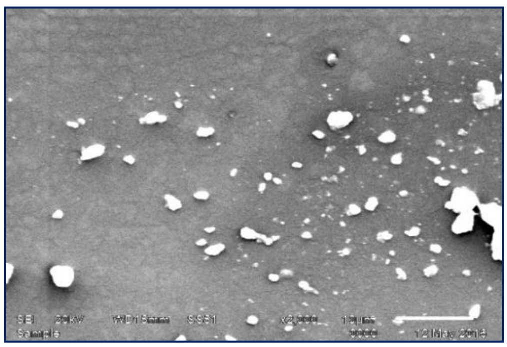

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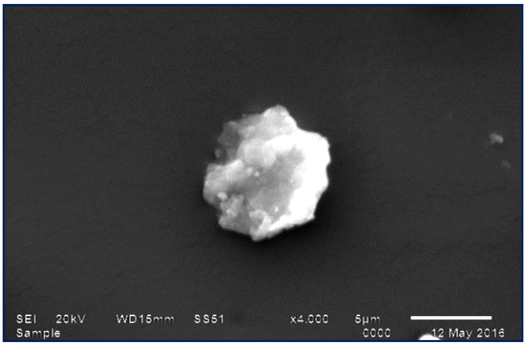

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Figure 2: SEM images at different magnifications (a.300X; b.2000X; c.4000X) of silver nanoparticles synthesized from Seeds of Gishta.

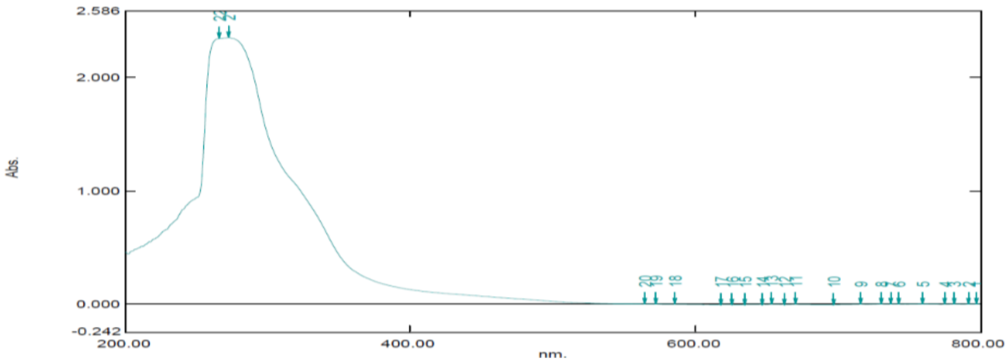


Figure 3: Ultra violet and Visible absorption spectrum of Ag nanoparticle of Ghista seeds

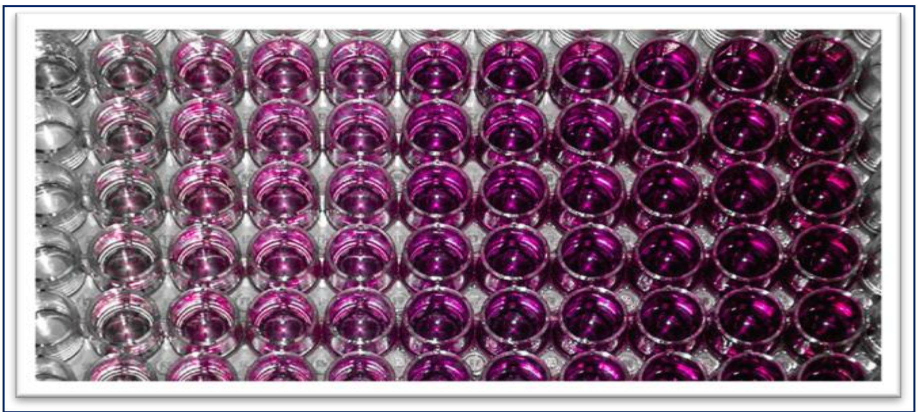


Figure 4 MTT Plate

2.8 Measurement of Caspase-3 activity

Caspase-3 activity was calculated by using ELISA method obtained by R&D System. The plates were conjugated with recombinant caspase-3 and provided with monoclonal antibody raised against caspase-3. All samples, standards, and reagents were prepared at room temperature then added to each well for 100 µl each and incubated at room temperature for 2 hours. After the incubation, each well was aspirated and washed 5 times. The wells were then added with conjugate, 100 µl and then incubated at room temperature for 1 hour and proceeded with washing the plates 5 times. Then, the plates were added with substrate solution of 100 µl and were incubated at room temperature for 30 minutes. Stock solution was added to each well and read the plates at 450 nm with correction wavelength of 540 nm. Caspase-3 was available in ng/ml which means the higher value was the better value of caspase-3.

2.9 Statistical analysis

The anticancerous data were analyzed by using 3 replicates and means were compared for any statistical difference through the least significant difference using the online software tool (statskingdom). If the p-value was less than 0.05, the means were substantially different.

3. Results:

3.1 Size of the peaks of the spectra

Peak size is another parameter that is accustomed to present the results of gas chromatography. The greater the scale of the peaks, the upper the concentration of a component of a sample.

3.2 Mass-to-charge Ratio (m/z)

This is the critical parameter that is derived from the mass spectrometry data. This ratio represents the mass of a given particle compared to the electric charge that it carries. The ratio is termed as m/z ratio. The spectrum of every component is exclusive. The mass spectra of the fragments are used as a puzzle to place together with the mass of the entire particle. This parameter is generally conclusive and provides an accurate method to spot a compound.

3.3 Synthesis and Properties of Ag-NPs

The Gishta seeds treated with AgNo3 solution and suspended till the colour change was observed from dark yellowish to brown (Image 1b) to confirm the Ag-NPs formation. Later different techniques were employed to learn their physicochemical properties. The stability and formation was studied with the help of UV-Vis spectroscopy and surface plasmon resonance band (SPR) analysis. The UV-Vis spectroscopy studies revealed an absorbance peak at 286nm and the multiple dispersed nature was indicated by its nature of broadening (Figure.2).

3.4. Morphological studies and elemental analysis of Ag-NPs

Morphological study of organic Ag-NPs was done using SEM microphotographs. We conducted SEM analysis to confirm and characterize the morphology with the help of S JEOL JSM-6610LV instrument. The results revealed that the particles predominantly exhibited a spherical shape, as evidenced in Image 3. A similar structural phenomenon of SEM image was reported by Chandran et al. 2006 and other research studies (Annamalai, A., et al., 2014; Erdogan, T., et al., 2016; Şeker Karatoprak, Gökçe, et al., 2016 and Deshi, Joseph., et al., 2016). The size of the silver particles ranges between 1 to 100 nm and predominantly between 30-70nm.

3.5 Cytotoxicity of Ag-NPs of Gishta seeds

Cytotoxicity of Gishta seed AgNPs was identified by MTT cytotoxicity assay, it is shown in Table 1. MMT assay showed how the sample could metabolize (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide). The Spectral analysis revealed cell viability.

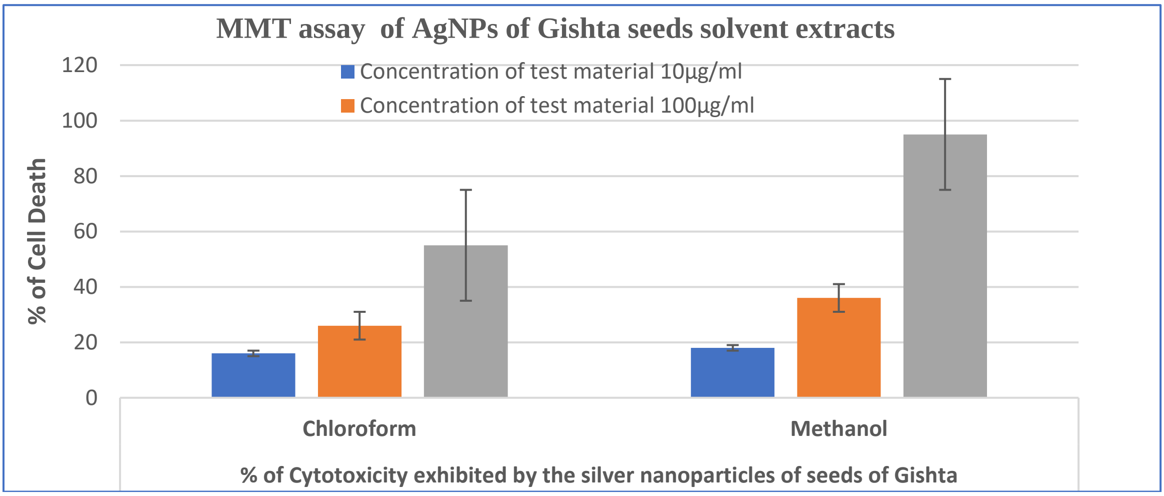
HepG2 cells exhibited cell death when they were treated with nano particles of Gista extract. The cell viability analysis is shown in Graph 1. Directly proportional relation was noticed between the cell death rate and the concentration of the Gishta nanoparticles.

Table 1 Cytotoxicity of AgNPs of Gishta seeds

S.No.	The concentration of test material (µg/ml)	% of cytotoxicity exhibited by the solvent extracts			
		Chloroform		Methanol	
		% Cell death	% Cell viability	% Cell death	% Cell viability
1.	10	16	84	18	82
2.	100	26	74	36	64
3.	1000	55	45	95	5

Cytotoxicity indicator: 50% or > cell death (Cytotoxic activity of Gishta mediated Ag-NPs of extracts against HepG2 (human hepatoblastoma cell lines)

The results indicate that the cytotoxicity of AgNPs from Gishta seeds is concentration-dependent, with higher concentrations leading to increased cell death in HepG2 cell lines. Methanol extracts displayed greater cytotoxicity compared to chloroform extracts, particularly at higher concentrations. This suggests that the solvent used for extraction plays a crucial role in the bioactivity of the nanoparticles.



Graph 1 MMT assay of Gishta mediated Ag-NPs of solvent extracts and cytotoxicity indicator (50% or > cell death) against HepG2 cells.

3.6 Caspase - 3 Activity

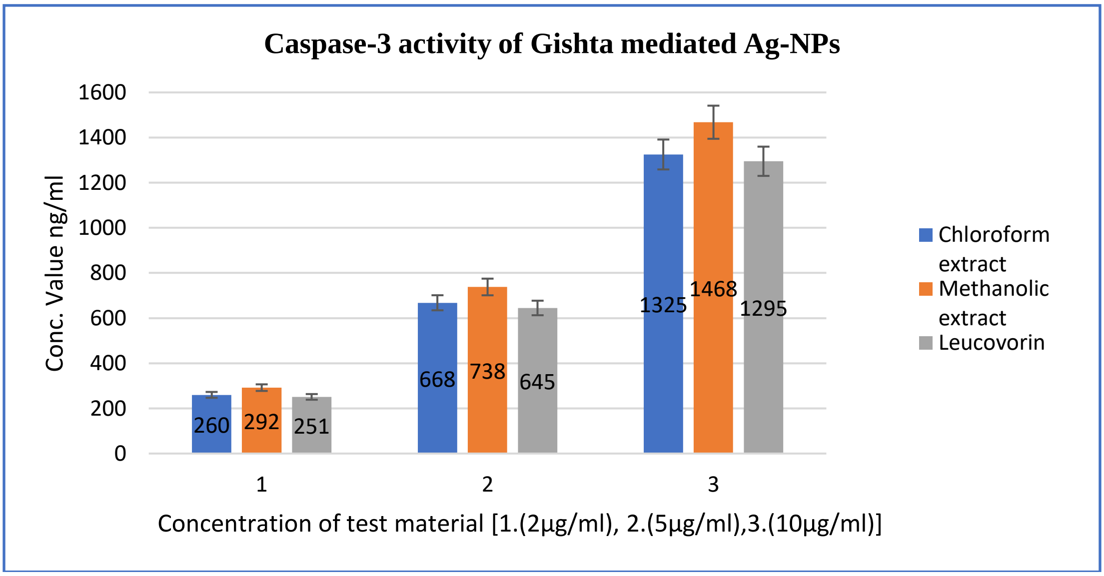
A comparative study of caspase-3 activity of chloroform extract (CE), Methanolic extract (ME) and leucovorin at different concentrations such as 2 µg/ml, 5 µg/ml and 10 µg/ml was done using Enzyme linked immune sorbent assay. Notable results were registered by ME extract at 10 µg/ml concentration with 1468 ng/ml value. The CE and leucovorin showed the values 1325 ng/ml and 1295 ng/ml respectively (Table 2. Graph 2). Gishta-mediated Ag-NPs of methanolic extracts upraised caspase-3 by 1.13 times, whereas chloform extracts upraised caspase-3 by 1.02 times.

Table 2 Caspase-3 activity of AgNPs of Gishta seeds

S.No.	The concentration of test material (µg/ml)	Caspase-3 activity exhibited by the solvent extracts		Standard leucovorin
		Chloroform extract	Methanolic extract	
		Conc. Value ng/ml	Conc. Value ng/ml	Conc. Value ng/ml
1.	2	260	292	251
2.	5	668	738	645
3.	10	1325	1468	1295

Caspase-3 activity of Gishta mediated Ag-NPs extracts against HepG2 (human hepatoblastoma cell lines)

The data indicates that both chloroform and methanolic extracts of AgNPs from Gishta seeds significantly enhance caspase-3 activity in a dose-dependent manner. Methanol extracts consistently exhibited higher levels of caspase-3 activation compared to chloroform extracts, suggesting that the solvent may influence the apoptotic efficacy of the AgNPs.



Graph 2. Caspase-3 activity of CE, ME, Leucovorin at three different concentrstions. Dominating result of ME at 10 µg/ml than Leucovorin and chloroform extracts.

4. Discussion:

New inorganic and organic based treatments should be developed to address the problems like cancer (Yee Y et al., 2009; Khan A. Q et al,. 2011).

It is clear that the plant extract mediated biosynthesis is amenable, effective, economical, ecofriendly and has no side effects for human therapeutic use. The green nanoparticles synthesis is a recently emerged viable method and a great alternative to the existing procedures (Chung IM, 2016; Ghotekar, S et al., 2019; Mukesh N. Kher et al., 2024) . The present analysis proved that the bio-moieties of seeds extracts helped in the making of Ag-NPs.

Human hepatoblastoma cells HepG2 (HB-8065) showed 95% death at 1000µg/ml and 36% death at 100µg concentrations of AgNPs. In biomedicine, drugs have been used for treatment in lower concentrations (E.J. Calabrese, 2014). As cancer tumors show microvasculature connection, the drug delivery with AgNps is possible by photothermal therapy to treat the cancer cells (Yao Y et al., 2020).

The cytotoxic value of Gishta mediated Ag-NPs extracts showed good results. Chloroform extracts inhibited cell proliferation up to 55% at 1000 µg/ml, whereas methanol extracts possesed 95% of cell profliferation inhibition at 1000 µg/ml. Through this research, the amount of IC-50 of Gishta mediated Ag-NPs extract in HepG2 (human hepatoblastoma cell lines) was seen. It is the required amount to inhibit cell proliferation to half extent in vitro (Bischof E, 2004; Muthu Irulappan Sriram et al., 2012). A study demonstrated that AnnomuricinE from Annona spp. inhibited HT-29 cells growth (Zorofchian Moghadamtousi S et al., 2015). It was found that the IC-50 of Annona spp. inhibited the growth of leukemia cells (Pieme C. A et al., 2014). In another study it was found that alcohol-soluble Annona spp. extract showed apoptotic nature (Astirin O. P., et al., 2013).

Anticancer nature of Annona leaves was studied by (Moghadamtousi S. Z et al., 2015). Increased caspase-3 activity in COLO-205 mediated through mitochondria was observed by (Niu G et al., 2011). Annona intervention could not elevate the action of caspase-3 (Mondal S. K et al., 2007). There was a decrease in procaspase-3 activity after 8 hours of administration of polyketides (acetogenin) (Yuan S. S et al., 2003). Caspase-3 mode of action in controlling cancer cells was studied by (Porter A. and Janicke R. U., 1999; Yuan SS et al., 2003). Present study has found that methanolic extracts of the plant under study has 1.13 times more caspase-3 activity than leucovorin and chloform extracts. Leucovorin efficacy in controlling cancer cells was revealed by Peters G. J et al., 2002 and Noordhuis P., et al., 2004.

The chemical nature of Annona spp. was studied and compared by (Kojima N and Tanaka T, 2009). Cytotoxicity of Annona spp.is attributed to bis-tetrahydrofuran ring system (Chang F et al., 2003; Qayed W. S et al., 2015). This result suggests the role of Gishta (Annona spp. of Ethiopia)) as combination therapy such as leucovorin and motivates the exploration of novel anticancer drugs using nanotechnology.

5. Conclusion:

This study highlights the promising anticancer properties of silver nanoparticles (Ag-NPs) synthesized using Gishta seed extracts. The observed cytotoxicity in all tested samples, coupled with the significant enhancement of caspase-3 activity compared to leucovorin, suggests that these nanoparticles may effectively promote apoptosis in cancer cells. However, further research is needed to isolate the active fractions responsible for enhancing caspase-3 functionality, which could provide deeper insights into their mechanisms of action.

Additionally, to fully realize the therapeutic potential of Gishta-mediated Ag-NPs, it is crucial to explore their biocompatibility and safety profiles for medical applications. These findings underscore the potential of green-synthesized Ag-NPs as a viable strategy for cancer treatment. Future investigations should focus on elucidating the molecular mechanisms underlying their cytotoxic effects and evaluating their clinical applicability in cancer therapy.

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