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Research Article

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In-vitro antioxidant and cytotoxic activities of ethyl acetate extract of *Holigarna ferruginea*

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ABSTRACT

Higher plants have long been used as traditional medicines to treat human ailments. Approximately 80% of people worldwide utilize plants as safe sources of medicines to heal human diseases via a totally new medicinal system. *Holigarna ferruginea* is an indigenous medicinal tree plant in the Anacardiaceae family. The plant has a wide range of physiologically active chemicals. GC-MS was used to screen phytochemical substances, while FTIR was used to identify functional groups. GC-MS study revealed 10 major bioactive phytochemical substances that belong to functional groups such as secondary amines, alcohols, ethers, esters, carboxylic acids, and anhydrides. These diverse active phytochemicals have been discovered to have a wide range of actions that may aid in the prevention of illnesses. Higher quantities of phytochemical substances were found in ethyl acetate extracts of leaves. As a result, the extract possesses anticancer and antioxidant activities against Humans Breast cell lines (MCF-7). The viability was reduced when the concentrations of the ethyl acetate extract of *H. ferruginea* leaves were increased and it may help in the discovery of an ideal therapeutic agent in novel drugs as well as nutritional supplements.

KEYWORDS: *Holigarna ferruginea*, GC-MS analysis, Antioxidant, cytotoxic activity

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INTRODUCTION

Cancer is one of the most dangerous diseases to human life, which manifests itself in more than 100 distinct forms as a consequence of chemical variations that take place inside cells. It ranks third on the list of top causes of death around the world, after cardiovascular illness and infectious disorders (Kelloff, 1999). In 2018, there were 18.1 million newly diagnosed cases of cancer and 9.6 million deaths attributed to the disease. There are 36 subtypes of cancer, the most common of which affect males in the form of colorectal, liver, lung, prostate, and stomach cancer, and strike women in the form of breast, cervical, colorectal, lung, and thyroid cancer (Bray *et al.*, 2018). Cancer can afflict both men and women. Research into ways of treating cancer has evolved into an entirely new field. There are both time-honored and cutting-edge approaches that can be taken in the fight against cancer. Cancer can be treated using a wide number of approaches, including chemotherapy, radiation therapy, and surgery, amongst others. Nevertheless, each and every one of them has a few disadvantages (Karpuz *et al.*, 2018). When traditional chemicals are used, there is a risk of experiencing side effects and toxicities (Nobili *et al.*, 2009). However, as the issue continues to exist, new methods are required for the control of diseases, particularly due to the failure of current chemotherapeutic methods. For this reason, there is a significant need for innovative approaches to the treatment and prevention of cancer in order to bring the mortality rate caused by this disease under control.

Now more than ever, herbal therapy is a reliable, non-toxic, and accessible way to treat cancer. Herbs have varied properties that make it possible for them to counteract the physiological effects of sickness (Khan *et al.*, 2019). And with their availability, low cost, and safety in usage, medicinal plants are frequently considered to be a possible source of natural chemicals and are therefore often used in traditional medicine systems. Approximately 75% of the world's population is indirectly or directly reliant on various plant species for the treatment of numerous deadly illnesses (Schippmann *et al.*, 2002). *H. ferruginea* is a huge semi-evergreen, and evergreen tree that is endemic to the Western Ghats, and it has black, acrid juice that can cause skin dermatitis (Srinivas *et al.*, 1987). Moreover, the other species in the genus *Holigarna* have some bioactive properties like antibacterial, antioxidant, and anti-inflammatory, and they have been reported by (Jadhav *et al.*, 2014; Ravi & Oommen, 2012; Adnan *et al.*, 2020; Uddin *et al.*, 2020; Panda *et al.*, 2020). This plant, however, has not been

researched for anticancer potential in women's breast cancer. As a result, we attempted to test the plant's efficacy against Humans breast cancer cell lines. The current study sought to assess preliminary phytochemical, *in-vitro* antioxidant, and cytotoxic potential actions against MCF-7 Humans breast cancer cell lines.

MATERIALS AND METHODS

Plant Collection and Crude Extraction

The plant specimens were collected from one of the evergreen forest sections in the Hassan District of Karnataka, India, which is located in the Bisle Ghat (12042′42″ N: 75041′17″ E). Figure 1 depicted the plant as well as the location where it was collected. Identification was confirmed by Dr. Y. L. Krishnamurthy, Professor of Botany, Kuvempu University, Karnataka, India and the specimen voucher number KUAB479 in the form of the herbarium was deposited in the department. The collected plant materials were powdered using a mechanical grinder after samples were cleaned and shade dried for 72 hours. An ethyl acetate solvent was used to extract the leaf powder. A 100 g dried leaf powder was weighed, placed in cheesecloth, and extracted with 500 mL ethyl acetate in the Soxhlet extractor for 48 hours or until the extract was clear. The extract was concentrated and stored in airtight vials in the refrigerator for future use.

Chemical and Reagents

DPPH (2, 2-Diphenyl-1-picrylhydrazyl), DMSO (dimethyl sulfoxide) was purchased from SigmaAldrich, Vitamin C (Ascorbic acid), MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), PBS (Phosphate buffered saline) was purchased from SigmaAldrich, and analytic grade Ethyl acetate were purchased and used.

GC-MS Analysis

The chemical composition of the *Holigarna ferruginea* leaves' ethyl acetate extract was examined by Gas Chromatography and Mass Spectrometry. A Thermo Trace GC Ultra Gas Chromatograph and a TSQ Quantum Mass Spectrometer were used to conduct the GC-MS analysis. Over the mass range of 40-500 Da, the mass detector was operated at 70 eV ionization energy, 0.132 s/scan in full scan mode. With a flow rate of 1 mL/min and a split flow of 25 mL/min, or a split ratio of 25, helium was employed as the carrier gas. The temperature of the transfer line was fixed at 200 °C. By comparing the compounds' mass spectra to reference mass spectra from various libraries, the NIST (National Institute of Standards and Technology), and information from previously published literature, the compounds were identified.

FT-IR Analysis of the Extract

The functional group of ethyl acetate leaf extract of *H. ferruginea* was analyzed using the FTIR system (Shimadzu Corporation, Japan) which was used to detect the characteristic peaks ranging from 400 to 4000 cm^{-1}. FTIR analysis may aid in the identification of biomolecules liable for biological actions. This qualitative examination observes the chemical bonding of samples by infrared light scanning. The form of the absorption spectrum profile in FTIR spectroscopy analysis displays unique peaks indicating the high concentration of specific types of chemical bonds, and different functional groups, such as alkanes, ketones, and amines, absorb infrared radiation of different wavelengths, allowing biomolecule identification (Palithya *et al.*, 2022).

Radical Scavenging Activity by DPPH

The free radical scavenging activity of the antioxidant activity of methanol plant extract was determined by using DPPH (2, 2-Diphenyl-1-picrylhydrazyl) (Sigma Aldrich) assay as described (Sharma & Bhat, 2009). About 1 mL of 0.002% 2, 2-Diphenyl-1-picrylhydrazyl was added to the equal volume of extract of different concentrations (1-1000 µg/mL) and standard vitamin C (Ascorbic acid), allowed to incubate at room temperature in dark for 30 minutes at 517 nm the absorbance was measured against blank (Ethyl acetate) by using a bio-spectrophotometer under the dim light source.

Percentage inhibition of the discoloration of DPPH by the extract was expressed as follows:

DPPH scavenging activity (%) = [(OD of Blank − OD of Sample)/OD of Blank] × 100

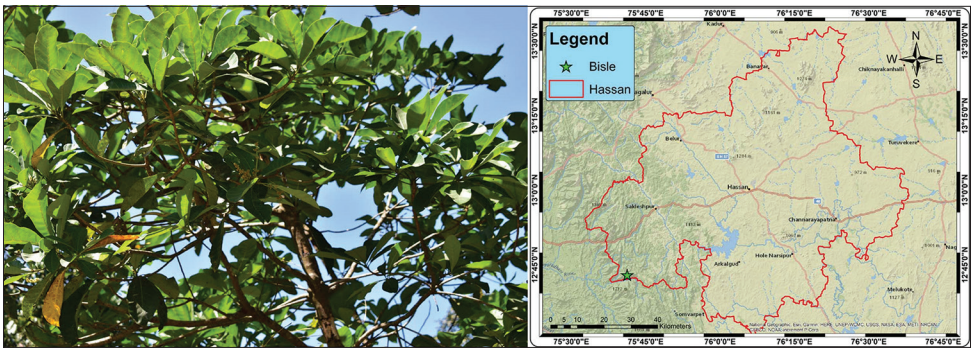

Figure 1: Habit and collection site of *H. ferruginea*

Cytotoxicity Activity by MTT Assay

The cell lines were washed twice with phosphate buffer saline (PBS) and centrifuged at 1500 rpm (Revolution per minute) for 3 minutes before being resuspended in an appropriate medium (medium with 10% foetal bovine serum) and plated in 96 well plates. The culture cells were incubated at 37 °C for 24 hours. The ethyl acetate extract of *H. ferruginea* and standard synthetic drug (Cisplatin) was subjected to *in vitro* cytotoxicity against MCF7 cell line by MTT assay as described by (Mosmann, 1983). The cell lines were washed twice with Phosphate Buffered Saline (PBS) and centrifuged, plated cell lines (3,000 to 10,000 cells/well) into 96 well microtiters, and allowed to incubate at 37 °C in a CO_2 incubator for 24 hrs. After the incubation period, 20 µL of MTT dye (5 mg/mL in PBS) was added. The absorbance was measured at 540 nm (or 540 nm concerning 630 nm) by using a multi-well plate reader.

Morphological Analysis

The changes in the morphological characteristics of MCF-7 cells after treatment with extract of *H. ferruginea* (Ethyl acetate extract) at different concentrations were observed under a light microscope and images were captured after the incubation of 24 hours and analyzed.

RESULTS AND DISCUSSION

Extraction and Yield

The biologically active compounds usually occur in low concentrations in plants. An extraction technique is that which is able to obtain extracts with high yield and with minimal changes to the functional properties of the extract required (Quispe-Candori *et al.*, 2008). Several studies have reported variations in the biological activities of extracts prepared using different extraction techniques. Therefore, it is necessary to select a suitable extraction method as well as a solvent-based. Here we found 16.56% of the total yield by ethyl acetate extract after evaporating the solvent and freeze-drying. The yield of extract was calculated by using the following formula:

Extraction yield (%) =Weight of the extract after evaporating solvent and freeze drying/Dry weight of the sample×100

GC-MS Analysis

GC–MS spectra of *H. ferruginea* extract revealed the peaks that indicated the occurrence of different compounds Figure 2. The spectral fingerprint of compounds identified using the data library and molecular weight, the compound names are listed in Table 1. Previously the compound l-(+)-Ascorbic acid 2,6-dihexadecanoate was reported as an anticancer and antioxidant compound from the *Bryonopsis laciniosa* fruits (Ramya *et al.*, 2015). Likewise, Squalene, Phytol, and Pentatriacontane have antioxidant and anticancer activities against different cancer cell lines (Sadiq *et al.*, 2018; Tarek *et al.*, 2020).

FT-IR Analysis of the Extract

Using the FT-IR spectra, the peak values in the infrared region allowed us to determine the functional groups of the active components included in the extract. FTIR spectra of *H. ferruginea* extract exhibited prominent peaks at distinct wavenumber (cm^{-1}) 1031.6, 1075.3, 1175.3, 1250.5, 1369.3, 1444.5, 1513.7, 1615.3, 2850.6, 2921.8, 3308.9 cm^{-1} shown in Figure 3. From the single bond region, the absorption peak at 3308.9 cm^{-1} was assigned to N-H stretching vibration which may be indicated the presence of secondary amines, representing the presence of a hydroxy group. The peaks at 2921.8 and 2850.6 cm^{-1} correspond to the C–H stretching vibration of alkanes. From the double bond region, the peak at 1615.3 cm^{-1} represents the C=C stretching vibration of α, β-unsaturated ketone. The peak at 1513.7 cm^{-1} correlates with the N-O stretching vibration of nitro compounds. From the fingerprint region (600-1500 cm^{-1}), the peak at 1444.5 cm^{-1} correlates with the C-H bending vibration of methylene. The peak at 1369.3 cm^{-1} correlates with the S=O stretching vibration of sulfonamide and the other peaks from fingerprint regions 1250.5; 1175.3; 1075.3 and 1031.6 cm^{-1} are majorly correlating with the C-O stretching vibration of Alcohols, Ethers, Esters, Carboxylic acids, and Anhydrides.

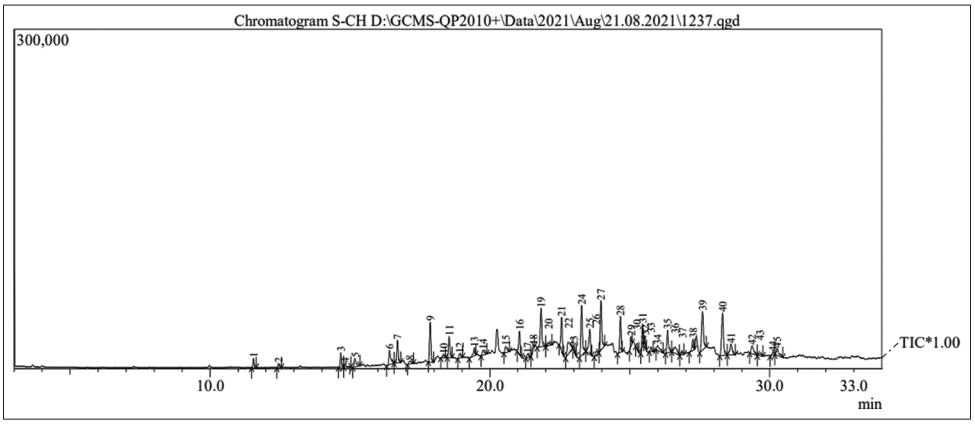

Figure 2: GC-MS profiles of ethyl acetate leaf extract of *H. ferruginea*

Table 1: Compounds present in the Ethyl acetate leaf extract of *H. ferruginea* using GC-MS analysis

S. No.	Peak Area %	Compounds	MF	MW
1.	2.5	l-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	652.9
2.	2.85	trans-6-Hexadecenoic acid	$C_{16}H_{30}O_2$	254.41
3.	5.06	Phytol	$C_{20}H_{40}O$	296.5
4.	2.91	1-Nonadecene	$C_{19}H_{38}$	266.5
5.	2.84	D- (-)-Quinic acid, tetramethyl ether, methyl ester	$C_{12}H_{22}O_6$	262.3
6.	5.81	Pentatriacontane	$C_{35}H_{72}$	492.95
7.	4.25	Tetracontane	$C_{40}H_{82}$	563.1
8.	3.36	d-Mannitol, 1-decylsulfonyl-	$C_{16}H_{34}O_7S$	370.5
9.	7.02	Pentatriacontane	$C_{35}H_{72}$	492.95
10.	3.92	Squalene	$C_{30}H_{50}$	410.72

DPPH Radical Scavenging Activity

The antioxidant activity of *H. ferruginea* was assessed by its capacity to scavenge DPPH free radicals. The DPPH radical-scavenging activity of *H. ferruginea* leaf extract was assessed and compared to that of ascorbic acid. The percentage of inhibition was calculated at different concentrations (1, 2, 4, 8, 10, 20, 40, 80, 100, 200, 400, 800, and 1000 g/mL) of the sample and standard (Figure 4). The greatest extract inhibition value was determined to be 1000 g/mL (82.33%), followed by 1 g/mL (14.944%), while the highest standard (Ascorbic acid) concentration was 1000 g/mL (83.997%) and the lowest was 1 g/mL (58.867%). Vitamin C is a potent antioxidant with the potential to donate a hydrogen atom and produce a rather stable ascorbyl-free radical (Pehlivan, 2017). In this study, the extract obtained from ethyl acetate solvent was studied for its antioxidant activity by using DPPH scavenging activity assays. The ethyl acetate extract was the most potent in terms of DPPH scavenging activity. This could be because this extract contained the highest level of phenolic compounds (Ruiz-Ruiz *et al.*, 2017). Those compounds possess powerful antioxidant activity and consequently protect the human body against oxidative damage through scavenging diverse reactive oxygen species, including hydroxyl radical anions (Chao *et al.*, 2014). Polyphenolic molecules are significant plant elements because of their hydroxyl groups, which allow them to scavenge free radicals. DPPH is a stable nitrogen-centered free radical that is often employed to assess the antioxidant scavenging properties of plant extracts or synthesized substances (Kalaivani & Mathew, 2010). Remarkably, the ethyl acetate extract of *H. ferruginea* exhibited a higher DPPH scavenging activity like ascorbic acid. These findings suggest that the acetate extract of *H. ferruginea* is a potential antioxidant agent for further drug development.

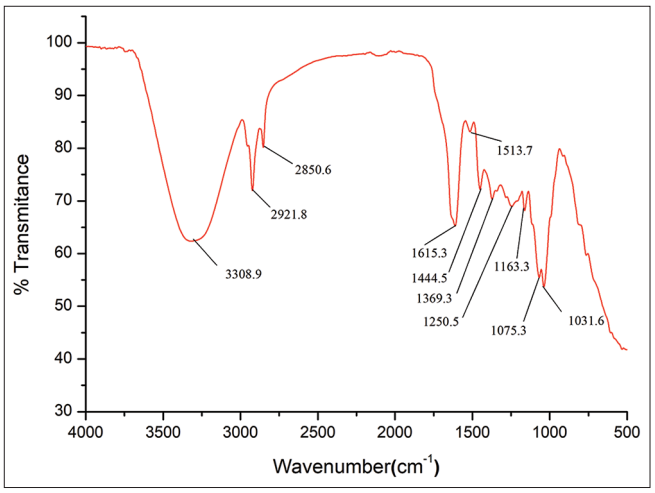

Figure 3: FT-IR spectrum of Ethyl acetate leaf extract of *H. ferruginea*

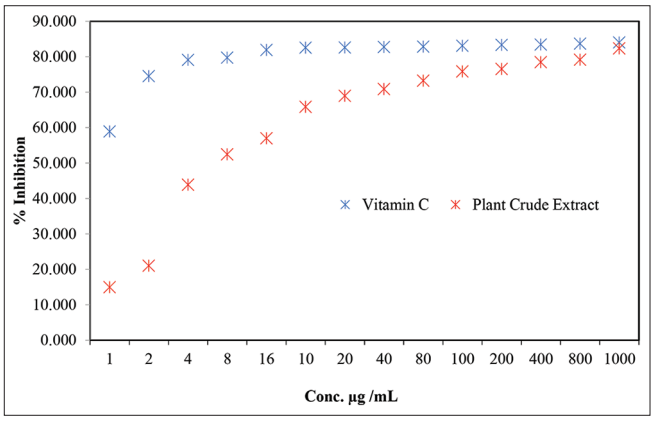

Figure 4: Antioxidant activity of Ethyl acetate leaf extract of *H. ferruginea* was compared with Ascorbic acid (Vitamin C)

The Cytotoxicity Effect and Morphological Changes on MCF-7

The cancer cell line (MCF-7) was treated with plant crude extract and synthetic drug cisplatin in a dose-dependent manner, the maximum cytotoxicity effect was exhibited at high concentrations. About 400 µg/mL of the extract showed the maximum reduction in the cell line viability of about 14.328%. Likewise, the cisplatin at about 500 µg/mL and 1000 µg/mL showed 6.01% and 3.31% of cell viability respectively shown in Figure 5. The medicinal plant extracts are effective against various cancer cells (Shokrzadeh *et al.*, 2010; Sodde *et al.*, 2015). Aydemir *et al.* (2015) found that plant extracts can cause cancer cells to die through the apoptosis or necrosis pathways. Breast cancer is progressively shifting into the category of long-lasting diseases in spite of the ongoing development and integration of current therapeutic techniques in medical technology. *H. ferruginea*, one of the medicinally important plants of the Anacardiaceae family has also been documented for its beneficial effects. However, the cytotoxic/antiproliferative effectiveness of *H. ferruginea* against the human breast cancer cell line (MCF-7) has yet to be investigated. As a result, the purpose of this study was to

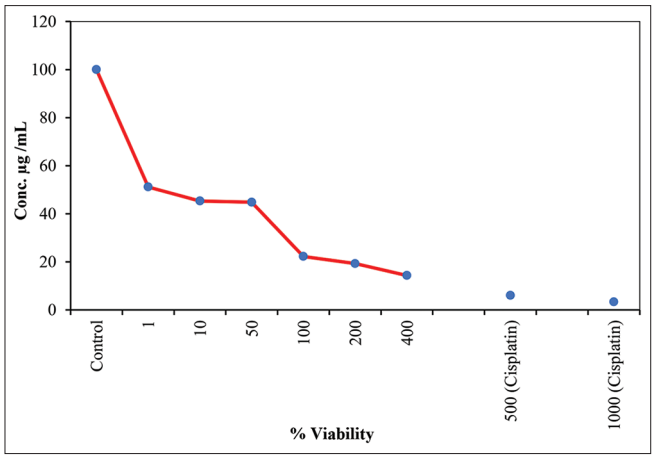

Figure 5: Cytotoxic activity of methanolic leaf extract of *H. ferruginea* against Humans breast cancer (MCF-7) cell line

evaluate the *in-vitro* cytotoxic effects of ethyl acetate extracts of *H. ferruginea* against the human breast cancer cell line (MCF-7). MTT assay and morphological analysis were used to assess the cytotoxic activity of ethyl acetate extracts of *H. ferruginea*. MTT tests were utilized in this investigation because they are simple, reliable, and sensitive, and have previously been used to assess the cytotoxicity and anticancer effects of plant extracts (Bacanlı *et al.*, 2017; Tihăuan *et al.*, 2020). In living cells, the MTT analyses evaluate the conversion of mitochondrial dehydrogenase enzyme into purple formazan (Mosmann, 1983). The results showed that the extract has dose-dependent cytotoxicity in MCF-7 cells. MTT experiments revealed that ethyl acetate extracts of *H. ferruginea* had a moderate cytotoxic impact on MCF-7 cells. A morphological study of MCF-7 treated with ethyl acetate extracts of *H. ferruginea* confirmed this finding. MCF-7 cells treated with ethyl acetate extracts of *H. ferruginea* extract lost their normal growth, became rounded, shrank, and inhibited cell proliferation, according to light microscopic pictures. All of these features indicated that the cells were experiencing apoptosis (Brady, 2004). The findings of this investigation demonstrated that ethyl acetate extracts of *H. ferruginea* at concentrations ranging from 1-400 µg/mL may considerably raise the level of LPO while decreasing the level of GSH. Previous research has also found that treatment of plant extracts enhanced LPO and lowered GSH levels in cancer cells (Al-Oqail *et al.*, 2019). It has also been shown that stimulating plant extracts can result in an increase in ROS production, which leads to oxidative and apoptotic cancer cell death (Marvibaigi *et al.*, 2016). The ethyl acetate extract of *H. ferruginea* treatment was may found to boost ROS generation dose-dependently in this investigation. These findings suggested that oxidative stress and ROS production may have a role in cell death produced by ethyl acetate extracts of *H. ferruginea* in MCF7 cells.

The Microscopic images of the MCF-7 cell line revealed the cytotoxic activity of *H. ferruginea* shown in Figure 6. The cell line was exposed to different concentrations of extract, and cell lines lost their normal growth and become rounded, shrinking and inhibited their growth. The plant extract of *H. ferrugi*nea showed moderate potentiality against the human breast cancer line.

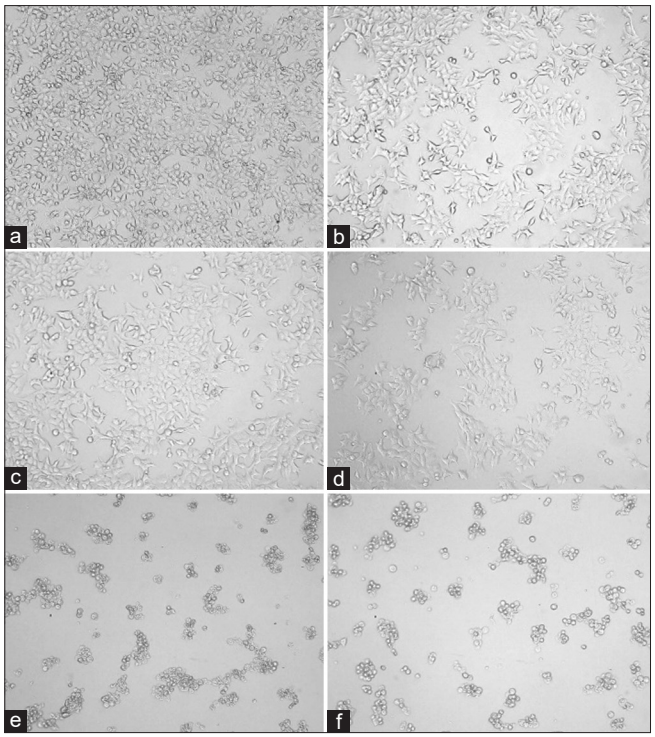

Figure 6: Morphological characteristics of MCF-7 cells after exposure to different concentrations: A-Untreated or Control, B-100 µg/mL, C-200 µg/mL, D-400 µg/mL, E-500 µg/mL of standard (Cisplatin) F-1000 µg/mL of standard (Cisplatin)

CONCLUSION

The use of natural or plant-based anticancer products is a useful tool to fight against cancer cells due to their few or no side effects. This study demonstrated that the ethyl acetate extract of *H. ferruginea* possessed promising antioxidant and cytotoxic activity compared to respective standard drugs. Moreover, when the extracts were screened for antitumor activity, MCF-7 breast cancer cell lines were significantly affected by different concentrations of ethyl acetate extract of *H. ferruginea* and is due to the presence of several active potent antioxidant and antitumor chemicals and making it an ideal therapeutic agent in novel drugs as well as nutritional supplements.

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