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K Thilagavathi
Department of Veterinary
Pathology, Veterinary College
and Research Institute,
Orathanadu (TANUVAS),
Thanjavur, Tamil Nadu, India

J Selvaraj
Department of Veterinary
Pathology, Veterinary College
and Research Institute,
Orathanadu (TANUVAS),
Thanjavur, Tamil Nadu, India

P Balachandran
Department of Veterinary
Pathology, Veterinary College
and Research Institute,
Orathanadu (TANUVAS),
Thanjavur, Tamil Nadu, India

V Ranganathan
Department of Veterinary
Pathology, Veterinary College
and Research Institute,
Orathanadu (TANUVAS),
Thanjavur, Tamil Nadu, India

T Lurthu Reetha
Department of Veterinary
Pathology, Veterinary College
and Research Institute,
Orathanadu (TANUVAS),
Thanjavur, Tamil Nadu, India

PC Prabu
Department of Veterinary
Pathology, Veterinary College
and Research Institute,
Orathanadu (TANUVAS),
Thanjavur, Tamil Nadu, India

Corresponding Author:
K Thilagavathi
Department of Veterinary
Pathology, Veterinary College
and Research Institute,
Orathanadu (TANUVAS),
Thanjavur, Tamil Nadu, India

Phytochemical analysis and anti-cancer effect of a polyherbal extract for mammary cancer by *in vitro* study

K Thilagavathi, J Selvaraj, P Balachandran, V Ranganathan, T Lurthu Reetha and PC Prabu

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Abstract

Currently, mammary cancer is a major health problem in human beings and dogs. Numerous studies have reported the anticancer effect of individual herbal plants for treatment of mammary cancer. This study focused on the phytochemical analysis and *in vitro* anticancer effect of polyherbal extract (Leaves of *Annona muricata*, *Azadirachta indica* and *Aegle marmelos* and seeds of *Nigella sativa*) for treatment of mammary cancer. Qualitative phytochemical analysis of polyherbal extract revealed the presence of various secondary metabolites like tannins, phenols, alkaloids, flavanoids, terpenoids, saponins, sterols, carbohydrates, and proteins. Quantitative analysis of polyherbal extract revealed highest concentration of flavonoids. Cells viability was assessed by *in vitro* study with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay which revealed the ability of polyherbal extract to inhibit the growth of breast cancer cells (MCF-7 cell line). The significant morphological changes were characterized by reduced cell density, rounded up cells, loses of contact with neighbouring cells and cell shrinkage. The results obtained from this study indicated that the polyherbal extract could be used for cancer treatment.

Keywords: Anti-cancer effect, MCF-7, phytochemical analysis, polyherbal extract, MTT assay

Introduction

Breast cancer is frequently diagnosed in women worldwide, leading to the second most common cause of death in women. Currently, breast cancer represents a serious public health problem in developing countries (Ullah, 2019) [27]. The incidence of mammary tumours in dogs is three times greater than humans. Mammary tumour occurrence is more common in intact females comprising up to 50 to 70 percent of all tumours (Moe, 2001) [19]. Many treatments directed against breast cancer are now available, some examples include; chemotherapy that can be used alone or in combination with surgery, radiotherapy, hormone therapy and targeted therapy (Tinoco *et al.*, 2013) [26]. However, these therapies do not absolutely reverse metastatic breast cancer and are laden with many other drawbacks including: chemotherapeutic drug toxicity on healthy cells, endometrial cancer risk and cardiotoxicity (Kumar, *et al.*, 2014) [13].

Phytochemicals act through multiple pathways that are involved in cancer progression, including cell cycle control, apoptosis, angiogenesis, and metastasis. Thus, their multimodal actions offer synergy with conventional therapeutic modalities, potentially improving therapeutic outcomes and reduces side effects (Aljabali *et al.*, 2025) [2].

Annona muricata Linn (common name "soursop") is a plant widely distributed in tropical regions. It is known to possess biological properties such as sedative, bactericidal, antiparasitic, anti-inflammatory, astringent, haemostatic, antispasmodic, anti-ulcer, anti-diarrhoeal, antitussive, hypotensive, antipyretic, galactagogue and anticancer (Maignien and Corosol, 2005) [17]. Administration of an ethanolic fraction of neem leaf (*A. indica*) inhibits progression of chemical carcinogen induced mammary tumourigenesis in rat models. Ethanolic fraction of neem leaf treatment was also highly effective in reducing mammary tumour burden and in suppressing mammary tumour progression even after the cessation of treatment (Arumugam *et al.*, 2014) [3]. *Aegle marmelos* leaf extract is found to be very effective against various tumour cell lines including breast cancer cell line MCF-7 (Michigan

cancer foundation) and MDA-MNB 231 (M.D. Anderson metastasis breast cancer) and it also has antineoplastic effects on the Ehrlich ascites carcinoma in Swiss albino mice (Lampronti, 2003; Jagetia *et al.*, 2005) [15, 9]. The extract of soursop leaf (*A. muricata*) is very active in inhibiting the growth of breast cancer cell line MCF-7, showing higher activity using MTT assay (Hasan *et al.*, 2020) [7]. When *Aegle marmelos* leaves extract treated MCF-7 cells was assessed by MTT assay using Dulbecco's Modified Eagle Medium. It inhibited the proliferation of MCF-7 cells, changed the cytoplasmic morphology, reduced the invasion and migration ability of MCF-7 cells and exhibited pro-apoptotic effects (Kumar *et al.*, 2023) [14]. The extracts and phytochemicals compounds of *Nigella sativa* extract containing its active component such as thymoquinone (TQ), carvacrol, and trans-anethole have been studied extensively for therapeutic potential against many diseases especially cancer. These bioactive compounds inhibit breast cancer cells and promote apoptosis. The apoptotic potential of *N. sativa* extract has also been demonstrated when studied in MDA-MB-231 and MCF-7 cell lines.

Most of the studies showed herbal treatment for mammary cancer by using single plant herbal extract. In order to find the new insight, present study is focused to evaluate the phytochemical analysis and anti-cancer effect of polyherbal extract (Leaves of *Annona muricata*, *Azadirachta indica* and *Aegle marmelos* with seeds of *Nigella sativa*) for mammary cancer treatment by *invitro* study.

Materials and methods

Leaves of *Annona muricata* were identified at Sidda Central Research Institute (Central Council for Research in Siddha, Chennai, Ministry of AYUSH, Government of India) and accession number was A22042329M dated 09.05.2023. Leaves of *Azadirachta indica* and *Aegle marmelos* were identified at Botanical survey of India, Southern Regional Centre, Tamil Nadu Agricultural University Campus P.O, Lawley Road, Coimbatore and the accession numbers are BSI/SRC/5/23/2024/Tech-131 dated 22.02.2024 and BSI/SRC/5/23/2024/Tech-130 dated 22.02.2024 respectively. The seeds of *Nigella sativa* were identified at Sidda Central Research Institute (Central Council for Research in Siddha, Chennai, Ministry of AYUSH, Government of India) and the accession number is N27022401S dated 13.03.2024.

Preparation of polyherbal extract

Fresh leaves of *Annona muricata*, *Azadirachta indica* and *Aegle marmelos* were collected and cleaned with water. These were dried in shade for 20-30 days. All the three leaves and seeds of *Nigella sativa* were individually crushed into powder using mixture grinder. The ethanolic extract of individual herbs were prepared by macerating the respective herbal powder in 70% ethanol for five days with intermittent shaking in the Department of Veterinary pathology, Veterinary College and Research Institute, Orathanadu. It was filtered using Whatman No.1 filter paper. The extracts were kept in rotary evaporator at 50 °C for three hours at the Department of Veterinary Pharmacology and Toxicology, Veterinary College and Research Institute, Orathanadu and dried to obtain a blackish-green semisolid substance. The dried extract was weighed and stored at -20°C in an air tight container until

further use. The individual plant extracts were mixed at the ratio of 1:1:1:1.

Phytochemical analysis

Qualitative phytochemical analysis to confirm the presence or absence of different phytochemical constituents like tannins, phenols, alkaloids, flavonoids, terpenoids, saponins, sterols, carbohydrates and proteins in the ethanolic extract of *A. muricata*, *A. indica* and *A. marmelos* leaves and seeds of *N. sativa* individually as well as in polyherbal formulation were done following standard procedures.

Quantitative phytochemical analysis of selective phytochemicals was analysed by using spectrophotometer. Total flavonoid content in polyherbal extract was determined according to the method of Zhishen *et al.* (1998). Tannin in polyherbal extract was estimated as per the procedures of The Ayurvedic pharmacopoeia of India 2008. Total phenolic content in polyherbal extract was determined as per Gupta *et al.* (2003). Saponin content in polyherbal extract was determined using the method of Obadoni and Ochuko (2001). The terpenoid content in polyherbal extract was determined using method of Indumathi *et al.* (2014).

In vitro study with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Cell viability assay using MCF-7 cell line was carried out using 10 per cent fetal bovine serum supplemented Dulbecco's Modified Eagle media. The cells were seeded in a 96-well flat-bottom microtiter plate at a density of 10⁴ cells/well and allowed to adhere for 24 hours at 37 °C in a CO₂ incubator. After 24 hours of incubation, culture medium was replaced with a fresh medium. Stock solution of polyherbal extract was prepared by mixing with Dimethyl sulfoxide. The cells were then treated with various concentrations (25, 50, 100, 200 and 400 µg/ml) of the polyherbal extract with three replicates for 24 hours at 37 °C in a CO₂ incubator. After 24 hours of incubation, culture medium was replaced with a fresh medium. Subsequently, 10 µL of MTT working solution (5 mg/mL in phosphate buffer saline solution) was added to each well and the plate was incubated for 4 hours at 37 °C in a CO₂ incubator. The medium was then aspirated, and the formed formazan crystals were solubilized by adding 50 µL of DMSO per well for 30 min at 37 °C in a CO₂ incubator. The plate was cover it with aluminum foil and mildly shaken for 10-15 minutes. Finally, the absorbance was read on a BIORAD iMax microtitre plate reader at 490 nm. The inhibition concentration IC₅₀ was calculated based on the OD value.

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treatment}}{\text{Absorbance of control}} \times 100$$

Results

Qualitative analysis

The results of qualitative analysis of the ethanolic extract of *A. muricata*, *A. indica*, *A. marmelos* and *N. sativa* as well as polyherbal extract are given in the Table 1. The leaves of *A. muricata* and *A. indica* were positive for tannins, phenols, flavanoids, terpenoids, saponins, sterols, and carbohydrates except alkaloids and proteins. The leaves of *A. marmelos* and seeds of *N. sativa* were positive for tannins, phenols, alkaloids, flavanoids, terpenoids, sterols, carbohydrates and

proteins except saponins. The polyherbal formulation were positive for all the constituents like tannins, phenols, alkaloids, flavanoids, terpenoids, saponins, sterols, carbohydrates, and proteins.

Quantitative analysis

The quantitative analysis of selective phytochemicals for polyherbal formulation are given in the Table 2. The concentration of phytochemicals were flavonoids, saponins, phenols, terpenoids, tannins and alkaloids were 13.65, 06.22, 03.89, 02.49, 01.05 and 00.40 (% w/w) in decreasing order respectively.

In vitro study with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cytotoxic effect of polyherbal extract in different concentration of 25, 50, 100, 200 and 400 µg/ml on MCF-7 cells was measured by MTT assay after 24 h of incubation. Cell viability reduced from 100% to 95, 87, 85, 73, 65% in response to dose dependent higher concentration of polyherbal extract from 25, 50, 100, 200 and 400 µg/ml respectively. The IC₅₀ value of polyherbal extract was estimated as 358.374 µg/ml (Fig.1). The morphological analysis of polyherbal extract treated MCF-7 cells was observed in inverted phase contrast microscope. The MCF-7 cells treated with polyherbal extract 25, 50, 100, 200 and 400 µg/ml compared with untreated (control) cells are given in the Fig. 2. The significant morphological changes were apoptosis which were characterized by reduced cell density, rounded up cells, loss of contact with neighboring cells and cell shrinkage and were observed in a dose dependent manner.

Discussion

Qualitative phytochemical analysis

The leaves of *A. muricata* and *A. indica* were positive for tannins, phenols, flavanoids, terpenoids, saponins, sterols and carbohydrates. These findings were in accordance with earlier reports of Daud *et al.* (2016) [4] who reported the presence of alkaloids, terpenoids, flavonoids, coumarins, steroids, fatty acids, phlobatannins, phenolics, tannins, and saponins in *A. muricata* leaves extract. The results were in agreement with Ravi *et al.* (2015) [23] who reported the phytochemical screening of ethanol extract of *A. indica* leaves and twigs revealed the presence of alkaloids, flavonoids, saponins, tannins, polyphenols and steroids. The leaves of *A. marmelos* and seeds of *N. sativa* were positive for tannins, phenols, alkaloids, flavanoids, terpenoids, sterols, carbohydrates. The results are in strong agreement with Veer and Singh (2019) [29] who reported the phytochemical screening of *A. marmelos* dried leaves revealed the presence of alkaloids, carbohydrates, flavonoids, phenols, saponins, steroids, and tannins. These results were similar to Umadevi *et al.* (2012) [28] who reported the presence of carbohydrates, alkaloids, flavonoids, tannins, terpenoids, saponins, quinones, glycosides, coumarins in the ethanolic leaf extract of *A. marmelos*. These results also were in agreement with Ahmad *et al.* (2014) [1] who reported that the phytochemical screening of methanolic extract of *N. sativa* seeds revealed the presence of carbohydrates, phenolic compounds, flavonoids, alkaloids, proteins, saponins, lipids, sterols and tannins. Qualitative phytochemical screening of combined polyherbal extracts from *A. muricata* leaves, *A. indica*

leaves, *A. marmelos* leaves and *N. sativa* seeds extract confirmed the presence of a wide range of secondary metabolites including tannins, phenols, alkaloids, flavanoids, terpenoids, saponins, sterols, carbohydrates, and proteins in ethanolic extracts. The detection of these bioactive compounds strongly supports the therapeutic potential of the polyherbal extract, as each plant component is already documented to contain similar phytochemical classes individually. These results align with published phytochemical reports for each species (Daud *et al.*, 2016; Ravi *et al.*, 2015; Umadevi *et al.*, 2012 and Ahmad *et al.*, 2014) [4, 23, 28, 1].

Quantitative phytochemical analysis

The quantitative analysis of selective phytochemicals of polyherbal extract in the current study contained higher concentration of flavonoids (13.65%) followed by saponin (6.2%). The results were in strongly agreement with Khanal (2021) [11] who reported the quantitative estimation of *A. indica* contained highest flavonoids percentage (13.8%) in leaves. Salsabila *et al.* (2021) [24] reported 2.3% of flavonoids in *A. muricata* leaves extract. It strongly supports the polyherbal extract as an anticancer agent. They also reported that flavonoid compounds are known for their antioxidant activity due to their compound structure which has many phenolic residues bound to the generic carbon ring framework. Phytochemicals are bioactive compounds produced naturally by plants and have great potential in human health and disease. These compounds possess antiproliferative, anti-oxidant, and immunomodulatory properties.

The flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized, according to chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. Numerous *in vitro* and animal model data suggest that flavonoids modulate important cellular and molecular mechanisms related to carcinogenesis, a multistep process involving the transformation, survival, proliferation, invasion, angiogenesis, and metastasis of the tumour cells. Flavonoids also act as protein kinase inhibitors for cancer chemoprevention. Recent studies show that flavonoids can bind directly to some protein kinases and then alter their phosphorylation state to regulate multiple cells signalling pathways in carcinogenesis processes (Umadevi *et al.*, 2012) [28]. Flavonoids are one of the most popular secondary metabolites possessing a variety of biological activities at nontoxic concentration. Dietary phytochemicals, mainly flavonoids hinder cell signalling responsible for multidrug resistance and cancer progression, primarily targeting cancer cells sparing normal cells. Such properties establish flavonoids as a potential candidate for synergistic therapy. However, due to low absorption and high metabolic rates, the bioavailability of flavonoids becomes a challenge. Such challenges may be overcome using novel approaches like derivatization and single or co-delivery nano-complexes of flavonoids with conventional drugs. It also highlights the application of flavonoids as a potential anticancer phytochemical class in combination with known anti-cancer drugs/nanoparticles (Hussain *et al.*, 2020) [8]. The flavonoids have been shown to possess a wide variety of anticancer effects: they modulate reactive oxygen species (ROS)-scavenging enzyme activities, participate in arresting the cell cycle, induce apoptosis, autophagy, and suppress cancer

cell proliferation and invasiveness (Kopustinskiene *et al.*, 2020) ^[12]. The biological potential of flavonoids, added to their bioavailability, cost-effectiveness, and minimal side effects, direct them as promising cytotoxic anticancer compounds in the optimization of therapies and the search for new drugs in the treatment of cancer, since some extensively antineoplastic therapeutic approaches have become less effective due to tumour resistance to drugs commonly used in chemotherapy. (Luna *et al.*, 2023) ^[16]. Among the phytochemicals, flavonoids are very effective in treating a wide range of diseases from cardiovascular diseases and immunological disorders to cancer. They scavenge reactive oxygen species (ROS), inhibit cancer metastasis, modulate the immune system and induce apoptotic or autophagic cell death in cancers. (Mir *et al.*, 2024) ^[18].

Cytotoxic effect of saponins was due to their ability to stimulate apoptotic process in tumour cells, usually through its intrinsic pathway. Moreover, non-apoptotic processes were also involved in saponin cytotoxic activity, such as cell cycle arrestment, autophagic cell death stimulation, inhibiting of metastasis and cytoskeleton disintegration (Podolak *et al.*, 2010) ^[22].

In vitro study with MTT assay

The *in vitro* cytotoxic activity of the crude extracts was determined from the mitochondrial activity of cell lines which represent the number of viable cells after the treatment, by using the MTT cytotoxic assay (Kaewpiboon *et al.*, 2012) ^[10]. In the present *in-vitro* MTT assay, the polyherbal extract composed of *A. muricata* leaves, *A. indica* leaves, *A. marmelos* leaves, and *N. sativa* seeds exhibited an IC₅₀ value of 358 µg/mL against MCF-7 human breast cancer cell lines. These findings were in accordance with earlier reports of Gavamukulya *et al.* (2014) ^[6] who reported the ethanolic extract of *A. muricata* leaves extract had IC₅₀ value of 335 µg/mL against MDA cell lines. They also reported that an integrated part of cancer cell development is the resistance to programmed cell death (apoptosis) and therefore re-establishment of apoptosis in cancer cells is a target mechanism for anticancer agents. Some plant derived products are known to selectively induce apoptosis in cancer cells, which represent the ideal property for successful anticancer agents. The current study showed the highly effective action of the ethanolic leaves extract of *A. muricata* and can be used in the management and treatment of cancer. This is in line with a study which showed that any extract had anticancer and cytotoxic activity if it had an IC₅₀ value less than 1 000 µg/mL after 24 h contact time, and that the smaller the IC₅₀ value of a test compound, the more toxic the compound was. Sharma *et al.*, (2014) ^[25] reported the antiproliferative effects of different concentrations of ethanolic neem leaf extract on MCF-7 cells, HeLa cells, and lymphocytes by MTT assay. MCF-7 and HeLa cells treated with increasing concentrations of ethanolic neem leaf extract ranging from 10 to 500 µg/mL showed a dose-and time dependent increase in cell death. In MCF-7 cells, the EC₅₀ (Effective concentration) was observed at 350 µg/mL after 72 h treatment with ethanolic neem leaf extract, whereas in HeLa cells, it was found to be 175 µg/mL in 48h. Mohammed *et al.* (2019) ^[21] reported that the treatment with different concentration of *A. muricata* leaves extract inhibited the

proliferation of MCF-7 cells in concentration dependent manner.

In the present study, the significant morphological changes were characterized by reduced cell density, rounded up cells, loses of contact with neighbouring cells and cell shrinkage indicating apoptosis in MCF-7 cell lines which is in accordance with Sharma *et al.* (2014) ^[25] who reported similar changes along with detachment from the substrate while studying the effect of ethanolic neem leaf extract in MCF-7 cell lines. The results were also in strong agreement with Kumar *et al.* (2023) ^[14] who observed similar morphological changes and stated that *Aegle marmelos* inhibited the proliferation of MCF-7 cells, changed the cytoplasmic morphology, reduced the invasion and migration ability of MCF-7 cells and exhibited proapoptotic effects. Fathima *et al.* (2023) ^[5] reported that the cells undergoing apoptosis displayed morphological changes such as rounded up cells that shrink and lose contact with neighbouring cells. Some sensitive cells detached from the surface of the plates in *A. marmelos* treated osteosarcoma cell lines. The results were also in strong agreement with Lampronti *et al.* (2003) ^[15] who observed *A. marmelos* treated human tumour cells showed cell shrinkage and subsequently lost contact with neighbouring cells.

Table 1: Qualitative analysis of the ethanolic extract of *A. muricata*, *A. indica*, *A. marmelos* and *N. sativa* and polyherbal extract

S. No	Phytochemicals	<i>A. muricata</i>	<i>A. indica</i>	<i>A. marmelos</i>	<i>N. sativa</i>	Polyherbal extract
	Tannins	+	+	+	+	+
	Phenols	+	+	+	+	+
	Alkaloids	-	-	+	+	+
	Flavanoids	+	+	+	+	+
	Terpenoids	+	+	+	+	+
	Saponins	+	+	-	-	+
	Sterols	+	+	+	+	+
	Carbohydrates	+	+	+	+	+
	Proteins	-	-	+	+	+
Key: (+): presence of phytochemicals (-): absence of phytochemicals						

Table 2: Quantitative analysis of selective phytochemicals in polyherbal extract

S. No	Phytochemicals	Polyherbal formulation (% w/w)
1	Flavonoids	13.65
2	Saponins	06.22
3	Phenols	03.89
4	Terpenoids	02.49
5	Tannins	01.05
6	Alkaloids	00.40

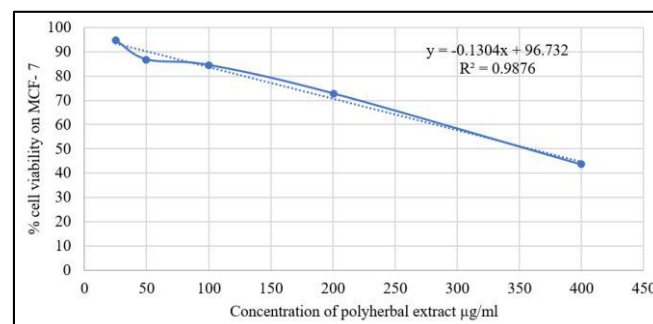


Fig 1: Cytotoxic effect polyherbal extract on MCF-7 cell lines.

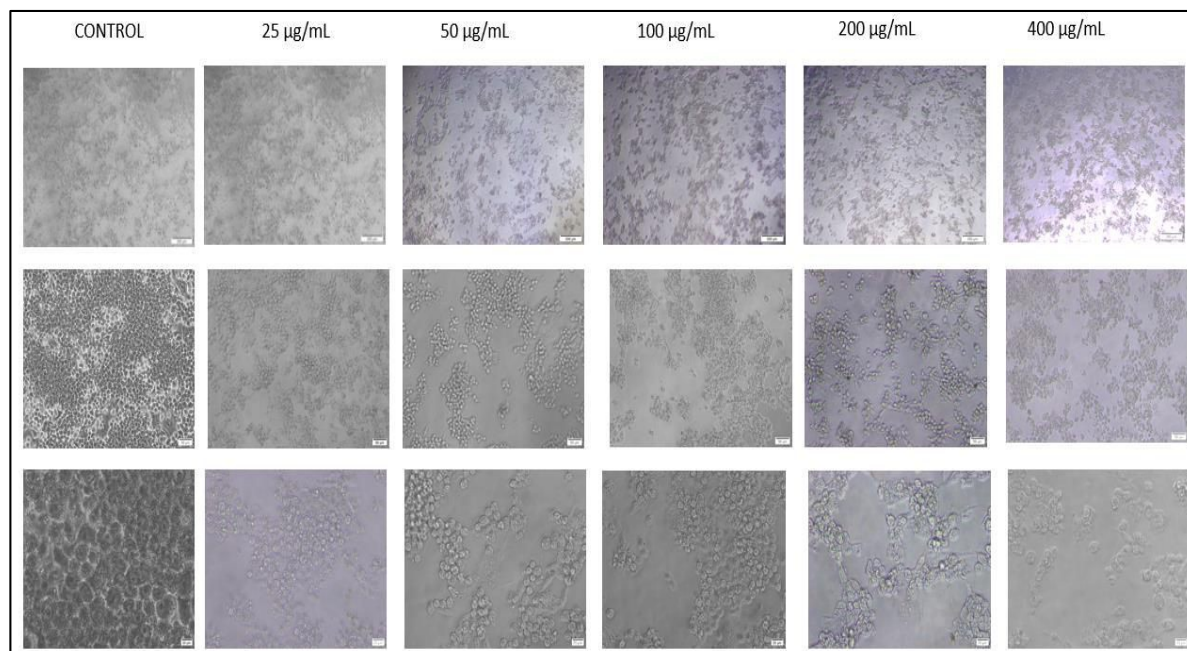


Fig 2: Morphological analysis of MCF7 cell line treated with polyherbal extract in different concentration compared with untreated (control) cells by inverted phase contrast microscope at 4X, 100X and 400X magnification. The significant morphological changes were apoptosis which were characterized by reduced cell density, rounded up cells, loses of contact with neighboring cells and cell shrinkage observed with increasing frequency in the order of 25, 50, 100, 200 and 400 µg/ml dose concentration.

Conclusion

Overall, the findings support the potential of the polyherbal extract (Leaves of *Annona muricata*, *Azadirachta indica* and *Aegle marmelos* and seeds of *Nigella sativa*) as a promising natural anticancer candidate for mammary cancer therapy. Further studies involving apoptosis assays, cell cycle analysis, gene expression profiling, and animal models are warranted to confirm its molecular mechanisms and therapeutic efficacy.

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