

***In Vitro* Anti-Metastatic and Antioxidant Activity of *Nicotiana glauca* Fraction Against Breast Cancer Cells**

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Abstract: Breast cancer is the most predominant cancer among the women. Drug discovery research and novel approaches to treat breast cancer has extensively been carried out worldwide. It goes without saying that herbal and traditional medicines play a significant role in the communities worldwide as it is considered as a source of effective remedies, especially against cancer. *Nicotiana glauca* (Solanaceae), or tree tobacco, is found in dry arid climates of North America, Africa, Europe and Australia. It is considered as a toxic plant in some places. However, *N. glauca* was reported to be anodyne and virucidal. It is a folk remedy for boils, headaches, piles, sores, wounds and cancer. In the present study, we investigated the cytotoxic, Anti-metastatic and antioxidant activities as well as quantifying the major chemical constituents of the active fraction of *N. glauca*. The dichloromethane fraction of N-hexane extract displayed selective cytotoxicity against the MCF-7 cells with (IC₅₀ 17.98 µg/mL). However, the extract did not show significant cytotoxicity against MDA (IC₅₀ 85.94 µg/ml) and normal cells (IC₅₀ 80 µg/mL). The apoptotic effects were investigated on mitochondrial membrane potential, cytoplasmic shrinkage and chromatin condensation. A mitochondrial apoptosis pathway was found to be involved in the cell death induced by the n-hexane fraction of *N. glauca*. Furthermore, the fraction demonstrated significant inhibitory effect on migration and invasion on MCF-7 cells. In addition, the fraction of *N. glauca* has shown high antioxidant potential. The results indicate that *N. glauca* possesses potent antitumor and antioxidant activities. Warrant further investigation is worthy to illustrate the potential chemotherapeutic agent.

Key words: MCF-7 (human hormone sensitive breast cancer cell line) • MDA-MB-231 (Human hormone resistant breast cancer cell line • NGAF (*Nicotiana glauca* active fraction)

INTRODUCTION

Over 200 types of cancer that harm the human body have been recognized. Cancer is the second leading cause of death in the entire world, surpassed only by heart disease [1]. It is reported in all ages and it is estimated that one out of three people in the world have a potential risk of being affected with this disease in his or her lifetime. The prevalence of cancer disease is expected to increase to 55% by 2020 [2]. Cancer is characterized by excessive and uncontrolled cellular growth followed by local tissue invasion and tumor metastasis [3]. Breast cancer is

considered the most common cancer among women with more than 23% incidence annually. It is the second most diagnosed cancer and fifth type leading to death [4-6]. There are two predominant types of breast cancer; the first one is age dependent and hormone resistance breast cancer (MDA-MB-231) which is primarily diagnosed before menopause. The second one with lower mortality rate commonly diagnosed after menopause called hormone sensitive breast cancer (MCF 7) [7]. Many breast cancer cell lines are used to evaluate the cytotoxic of new anticancer agents. The most commonly used ones called invasive ductal carcinoma includes: MCF- 7, BT20,

MDA-MB-435, T47D and ZR75.1. Others called adenocarcinomas such as MDA-MB-231, MDA-MB-468 and SkBr3 [8]. Using plants as remedies to cure diseases passed from one generation to another throughout the history of mankind [9]. In the present time, almost half of the population in the whole world are relying on the herbs as a sources of medicines [10]. Between 1981-2002, Over 60% of anticancer drugs approved by FDA were originally isolated from nature [11]. *N. glauca* is prevalent throughout Europe, Africa, North America, Australia, New Zealand and Mediterranean countries [12]. It is extensively prescribed by traditional healer as antibacterial, anti fungal, antiviral and anti-inflammatory medicines [13]. Its biological activity has been studied by Ibrahim [14] Previously, we have reported the cytotoxic efficacy of n-hexane extract against MCF7 [15]. The aim of this study is to evaluate Anticancer and Antioxidant Activities of *Nicotiana glauca* against Breast Cancer Cells.

MATERIALS AND METHODS

Cell Lines and Media: All the cell lines used were purchased from American Type Culture Collection (ATCC), USA. Two cancer cell lines as well as one normal cell line were used in this study. MCF-7, human hormone sensitive and invasive breast cancer cell line; Human hormone resistant breast cancer cell line (MDA-MB-231) were maintained in DMEM and normal mouse fibroblast (3T3) cell line was maintained in F-12K medium. The media was supplemented with 5% heat inactivated fetal bovine serum and 1% penicillin/streptomycin [16].

Plant Collection and Authentication: *N. glauca* material was collected during the period of spring season of 2013 from the Botanical garden, Kassala- Eastern Sudan. The taxonomic authentication of the plant was carried out by Dr. Wail Alsadig at The Medicinal and Aromatic Plants Research Institute, National Center for Research, Sudan. Voucher specimens (voucher references numbers: MAPRI/NB-53b) was deposited at the herbarium of the institute endangered or protected species.

Extraction and Fractionation: The extraction process was done as described before [15]. Briefly, the stem of *N. glauca* was cleaned and dried in oven. The plant material was grinded using small blender. The material was added in small skills (around 50 g) to 200ml n-hexane solvent. Sequential extraction was performed after n-hexane

followed by methanol. The dried residual extract was immersed in water for overnight to obtain water extract. a big amount (7g) of the n-hexane extract was subjected to vacuum liquid chromatography which was performed on column (10 × 7 cm) packed with silica gel of particle size (0.04-0.06 mm, 60-120 mesh). Solvent mixtures of n-hexane, dichloromethane and methanol were used in sequence of increasing polarities. Eluents of 100 mL each were collected and monitored by thin layer chromatography (TLC). The eluents with similar TLC chromatogram pattern were pooled together to obtain six main fractions. All extract and fractions were concentrated under reduce vacuum using BUCHI Rotavapor® (Germany) to get dried fractions subjected to bioassay [17].

Gas Chromatography-Mass (GC-MS) Spectral Analysis:

Quantitative chemical analysis of *N. glauca* active fraction *NGAF* was conducted using Agilent 6890 gas chromatography instrument equipped with Agilent 5973 Mass spectrometer. The assay conditions were as follows: HP-5MS capillary column (30 m × 0.25 mm ID × 0.25 μm, film thickness); held at 70°C for 2 min, raised to 285°C at a rate of 20°C/minute and held for 20 min; 285°C for MSD transfer line heater; carrier helium at a flow rate of 1.2 mL/minute; 2:1 split ratio. 1 μL solution of SF-1 in chloroform (10 mg/mL) was injected automatically. Scan parameter low mass: 35 and higher mass: 550. The constituents were identified by comparison with standards using NIST 02. A total ion chromatogram (TIC) was used to compute the percentage of the identified constituents [18].

Studies of Cytotoxic and Apoptotic Properties

Viability Testing by MTT Test: Cells were seeded (5000/100 μL) using the specific media in 96 well plate. After incubation for 24 hours, treatment of different concentration of *NGAF* was applied in 100 μL media. Cells were kept in oven 37°C for 48 hours. After adding 20 μL of MTT reagent (5 mg/mL) to each well, the plate was incubated for additional 3 h. Subsequently, the media was aspirated carefully and 100 μL dimethyl sulfoxide (DMSO) was added. After 10 min incubation at 37°C, optical density (OD) of the violet color was measured at A570 and a reference wavelength of 650 nm by a microplate reader (Thermolab Systems 354, Finland) The IC50 was calculated according to the equation:

% of cell viability = ((OD Sample – OD Blank) / (OD Control – OD Blank)) * 100 [19]

Changes in Mitochondrial Membrane Potential and Chromatin Condensation: MCF 7 cells were cultured in 24-well plates MCF 7 cells. After overnight incubation, cells were treated for 6, 24 h with different concentration of the compound and 1% DMSO as negative control. The cells were fixed in 4% paraformaldehyde for 20 min before staining with Hoechst stain 33342 (10µg/ml in PBS) rhodamine 123 at 1 µg/mL for 30 min. Nuclear condensation and cytoplasm shrinkage were examined under a fluorescent microscope (Olympus, Japan). The apoptotic index was calculated as the percentage of apoptotic cells compared to the total number of cells [20].

Anti Tumorigenicity

Cell Migration: The capability of *NGAF* to crab the metastasis was investigated *In vitro* using Wound healing assay as described before by Liang *et al.* [21]. Cells (1 x 10⁶) was seeded in 6 well plates. After 24 hours, an attached monolayer of MCF7 cells was performed. The monolayer was scratched by 100 µL micropipette tips to form a wound. Different concentrations of *NGAF* were applied. Pictures of the wounds were taken in different time (0, 6, 12 hours) and the widths of the wounds were measured by Leica Quin software. The resulted distances were reported as percentage of inhibition of migration in comparison to the mean distance for the negative control; it was calculated from the following formula:

$$\% \text{ of inhibition of migration} = (1 - (W_s / W_c)) \times 100$$

W_s = Distance of width travelled by cells

Antioxidant

DPPH Radical Scavenging Assay: DPPH radical scavenging activity of *NGAF* was estimated as described before [22]. The absorbance of the remaining amount of DPPH was measured using UV spectrophotometer. Ascorbic acid was used as reference standards.

ABTS Radical Scavenging Assay: Radical scavenging activity was measured by the method described by Re *et al.* [23]. Briefly, adding 180 µl of the final ABTS solution to 20 µl of the *NGAF* (in serial six concentrations). The plate was incubated for 6 min and was readed at 734nm. Ascorbic acid was used as reference standards.

Total Flavonoid Content: Total flavonoid content of *NGAF* was conducted as described by Al-Mansoub, Asmawi and Murugaiyah [24] using spectrophotometric methods. Briefly, the fraction or standard (100 µL) were

mixed with 20 µL of (10%, w/v) aluminum chloride, 300 µL methanol, 20 µL of 1 mol L⁻¹ sodium acetate and 560 µL distilled water. Subsequently, the plate was incubated for 30min and absorbance was measured at 415 nm. quercetin was used as a reference standard.

Ferric Reducing Antioxidant Power (FRAP) Assay: Antioxidant potential of *NGAF* by (FRAP) assay was measured using the method described by Benzie and Strain [25]. Using 96 well plates, 150 µL of (acetatebuffer, TPTZ, HCl and FeCl₃) was added to 50 µL of *NGAF*. The plate was incubated for 8 min. (FeSO₄.7H₂O) was used as reference.

Total Phenolic Content: To determine the total phenolic content of *NGAF*, Folin–Ciocalteu reagent was used as described by Tapan Seal [26]. The wavelength used was 765nm. the concentration was calculated according to the equation.

$$\text{Absorbance} = 0.0105 \text{ gallic acid } \mu\text{g} + 0.0056 R^2 \quad \frac{1}{4} \quad 0.9998$$

RESULT AND DISCUSSION

Gas Chromatography-Mass (GC-MS) Spectral Analysis: The *NGAF* was subjected to GC-MS analysis to quantify the major chemical constituents and molecular weight. The GC-MS chromatogram is shown in Figure 1 while GC-MS data such as retention time, % area peak, molecular formula and molecular weight obtained for the major phytoconstituents are given in Table 1. Chemical composition of the *NGAF* was identified using the NIST library. Accordingly, the GCMS analysis revealed that the mixture of *NGAF* was composed of various polyphenols and aromatic compounds. The quantitative analysis of *NGAF* showed that it contains major anticancer compounds which may explain the anti-proliferative efficacy of the fraction. Among the compounds inside the plant, Phytosterols and plant sterol which are equivalent to cholesterol in animal in slight structurally differences. Phytosterols has three main forms are b-sitosterol (SIT), campesterol and stigmasterol. It was proved as chemopreventive for colon, prostate and breast cancers [27]. Campesterol is considered the most common phytosterol isolated from nature. It is structurally resemble the plant sterol. Its biological activity has been reported as anti-carcinogenic and cholesterol-lowering agent [27, 28]. It has potent anti-angiogenesis by significantly inhibiting bFGF-induced proliferation and tube formation

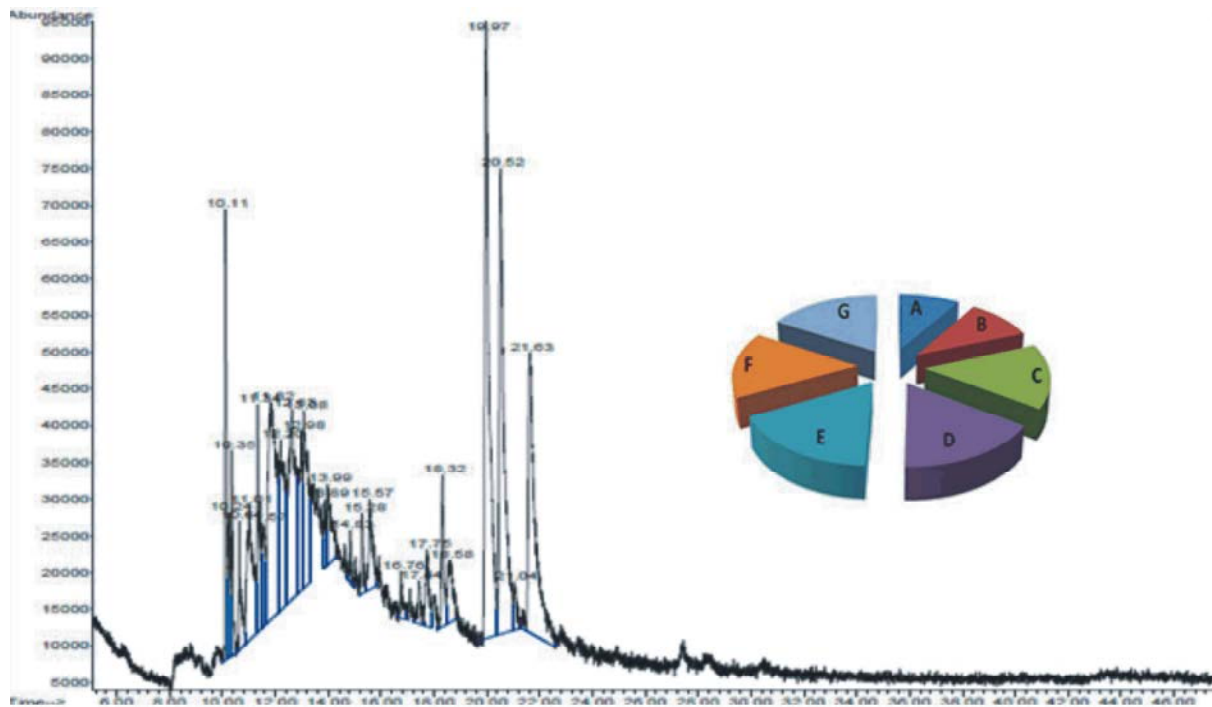
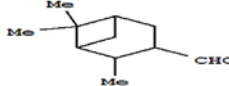
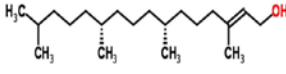
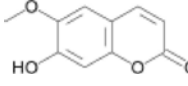
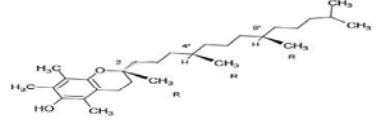
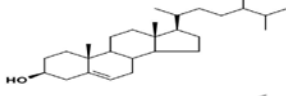
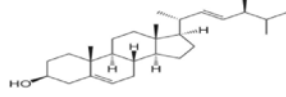
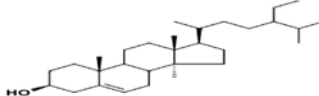


Fig. 1: Shows the chemical characterization of *NGAF* on chromatogram of GC-MS. The pie charts depict the relative chemical compositions. The details of the peaks are given in the Table 1.

Table 1: GC-MS quantitative estimation of phytochemicals of *NGAF*

peak	Ret time (min)	Phytoconstituents	Mol formula	Mol weight	Structure
A	10.11	Bicyclo[3.1.1]heptanes	C ₁₀ H ₁₈	138	
B	10.36	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	C ₂₀ H ₄₀ O	296	
C	13.2	Scopoletin	C ₁₀ H ₈ O ₄	192.16	
D	18.32	D, .alpha.-Tocopherol	C ₂₉ H ₅₀ O ₂	430	
E	19.97	Campesterol	C ₂₈ H ₄₈ O	400	
F	20.52	Stigmasterol	C ₂₉ H ₄₈ O	412	
G	21.63	beta.-Sitosterol	C ₂₉ H ₅₀ O	414	

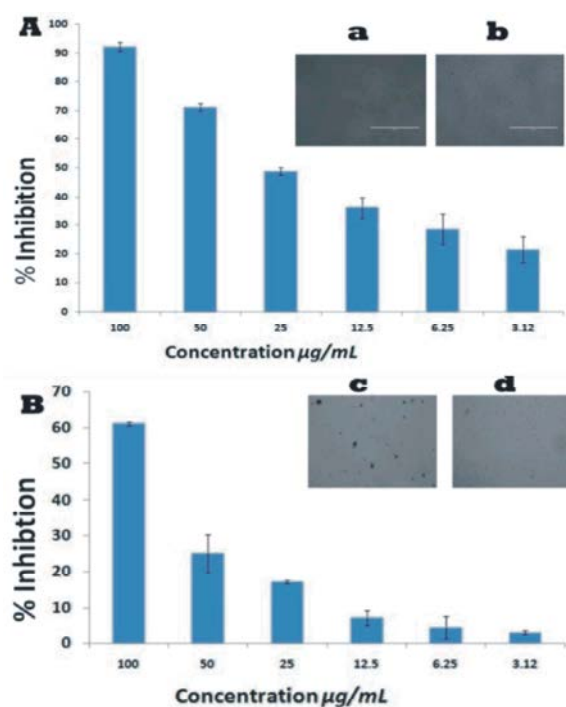


Fig. 2: Dose-dependent anti-proliferative activities of *NGAF*. (A) Depicts the percentage of proliferation inhibition of *NGAF* in MCF-7 cell line. (a) Photomicrographic image of treated MCF-7 cells whereas (b) for untreated cells. (B) Illustrates % inhibition of *NGAF* on MDA. (c) Photomicrographic image of treated MDA cells whereas (d) for untreated cells with *NGAF*.

[29]. Previously, stigmasterol has showed a significant protective effect against EAC bearing mice as well as antitumor and antioxidant activity [30]. Scopoletin has been reported as strong antiangiogenic agent by inhibiting the activation of ERK1, VEGF-A FGF-2, PGE₂ and TNF- α [31]. It crabs the metastasis of endothelial cells and induces cell cycle arrest and apoptosis. it was isolated from many herbal medicinal plants such as *Castanea crenata* [32]. *Crossostephium chinensis* [33]. *Nicotiana glauca* [14]. D, alpha.-Tocopherol is one of the main form of Vit-E, its anti-proliferative activity against vascular smooth muscle cells, Balb c/3T3 fibroblasts, retinal neuroepithelial cells and neuroblastoma cells has been studied [34].

The Cytotoxic effect of *NGAF* against Brest cancer Anti-proliferative effect of *NGAF* was tested against two Brest cell lines and one normal cell line using MTT assay. (IC₅₀) values were calculated for each cell line. *NGAF* has shown a potent anti-proliferative effect against MCF-7 with IC₅₀

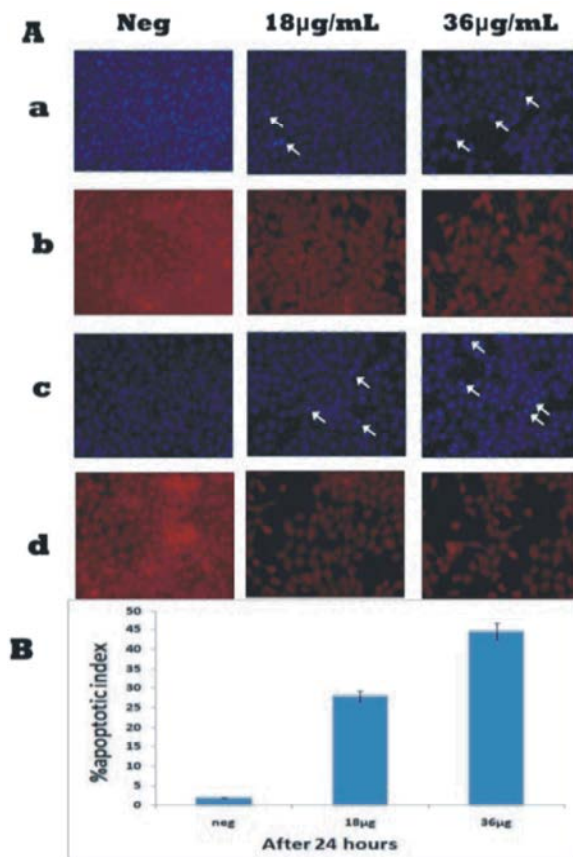


Fig. 3: A) The photomicrographs of MCF-7 using both Hoechst 33258 stain (a-c) and rhodamine 123 (b-d). Treatment the cells for 6 and 24 hours (b-d) respectively show disrupt of mitochondrial potentials and loss of the rhodamine 123 fluorescent signal. The ability of *NGAF* to distribute the nucleus in cytosol and form nuclear condensation was shown in (a-c) after staining the cells with Hoechst 33258. The untreated cells showed intact cells comparing with the treated one.

B) Graphical representation of percentage of apoptotic indices. The apoptotic index for each test group was expressed as a percentage of the ratio of number of unstained cells to the total number of cell in 10 different microscopic fields after 24 hours treatment with *NGAF*. Values are presented as mean \pm SD (n = 5)

= 17.98 μ g/mL. However it has demonstrated a poor cytotoxic effect against MDA with IC₅₀ = 85.94 μ g/L (Figure 2) and not significant anti-proliferative effect against normal mouse fibroblast (3T3) with IC₅₀ = 80 μ g/mL.

Table 2: Antioxidant activities of *NGAF*

Test Samples	IC ₅₀ values in µg/ml				
	DPPH	Fra-B	TFC	TPC	ABTS
<i>NGAF</i>	86.65	528.61	20.79	108.18	67.58
Vit.C	8.96±0.23	566.89	--	--	--

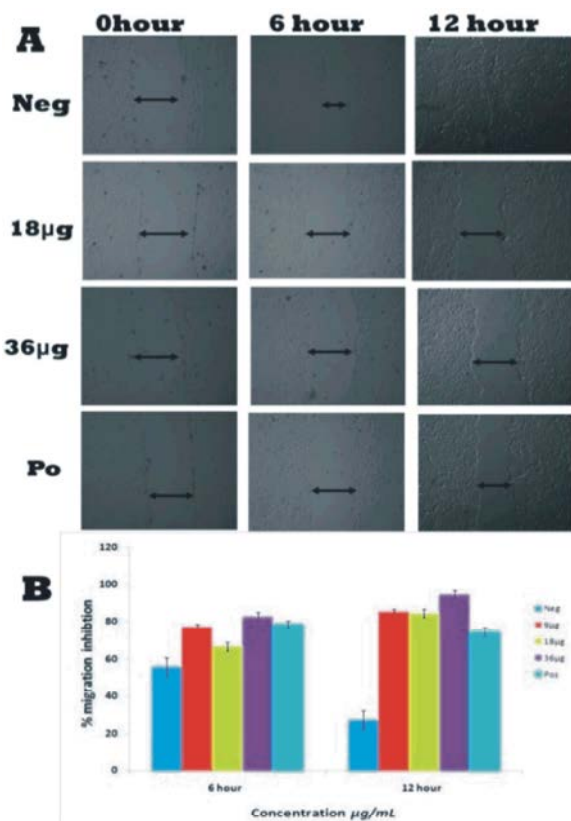


Fig. 4: A) photomicrographs of the created wounds with and without treatments of *NGAF*. The negative showed complete closeness of the wound after 12 hours. However, the treated wound remained open even after 12 h incubation.

B) Graphical representation of the time and dose and time-dependent inhibitory effect of *NGAF* on migration of MCF-7 (Values are in mean ± SD, n = 6).

Changes in Mitochondrial Membrane Potential and Chromatin Condensation: It is well reported study that apoptotic cells go through a series of morphological changes namely, membrane blebbing, nuclear condensation, DNA fragmentation and chromatin dissolution [35]. The apoptotic effect of the *NGAF* on the morphological modification and nuclear condensation of breast cancer cell (MCF 7) was studied using a lipophilic cationic dye rhodamine 123 and Hoechst 33342

stain. The uptake of rhodamine 123 demolishes when the mitochondrial membrane potential decreased. In the present study, *NGAF* displayed a high intensity fluorescent in the negative control which is a sign of a huge number of intact cells. However, treating cells with *NGAF* for 6 and 24 hours in two different doses (18, 36 µg/mL) showed a loss of mitochondrial potentials and the fluorescent signal. On the other hand, staining the treated cells with Hoechst 33342 illustrate a shrunken, crescent-shaped and condensation in the nuclei with irregular distribution of chromatin throughout the cytoplasm. The apoptotic index was calculated as described before [36]. The apoptotic index for 24 hour treated cells with two concentrations (18, 36 µg/ml) was 27.4 and 44.4% respectively (Figure 3).

Cell Migration: Cell migration is considered one of the critical step in the progress of angiogenesis, it resemble the metastasis process in the body. The metastatic inhibitory effect of *NGAF* was studied using three concentrations (9, 18 and 36 µg/ml). 5-Fluorouracil was used as positive. Treating the scratched cells with *NGAF* in three different doses (9,18 and 36 µg/ml) for 12 hours showed potent inhibitory migration effects (85.4, 84.5 and 94.5%) comparing to the negative and positive control (27.2 and 74.5%) respectively. Figure (4)

Antioxidant: Five antioxidant assays were conducted to represent the radicals and antioxidant activities of *NGAF*. The fraction showed high total phenolic content (108.18 µg/ml) and slight content of total flavanoid contents (20.8 µg/ml). The presence of total phenolic and flavonoids in plants are responsible of many biological activities including free radical scavenging and antioxidant effects through reducing hydrogen donors, free radical scavengers, singlet oxygen quenchers and metal chelators[37].in addition, A strong relationships between the content of phenol and antioxidant properties has been reported by its scavenging activities of hydroxyl group[38]. In the present assay, *NGAF* showed a weak DPPH and ABTS radical scavenging activity comparing with ascorbic acid. On the other hand, Fra-B has demonstrated similar scavenging activities between the fraction and positive control. In our study, the antioxidant

power of *NGAF* may correlate with its high amount of phenols. GCMS result showed a big content of antioxidant potent compounds which lead the fraction to be effective as antioxidant (Table2).

CONCLUSION

In our study, we demonstrate the apoptotic and anti-metastatic properties of *Nicotiana glauca* fraction against breast cancer cell lines as well as the antioxidant and the active ingredients responsible on its activity. A future investigation is worthy to illustrate the potential chemotherapeutic agent.

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