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In vitro Assessment of Antioxidant and Anticancer Potential of Flavonoid Glycosides of *Glinus oppositifolius* (L.) Aug. DC.

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Development of unbalanced state of free radicals and their elimination in the body is the reason for increase in different types of chronic diseases including cancer. A better natural supplement to play role of antioxidants to combat the free radicals and to reduce the risk of cancer is in high demand. This current research work focused on identifying such a potent phytocompounds from *Glinus oppositifolius* (L.) Aug. DC. The active compounds of the aerial parts of the plant have been extracted using methanol and the phytochemical analysis was carried out using qualitative tests, FTIR and GC-MS. The plant extract found to contain range of phyto compounds with good amounts of flavonoid and xylofuranoside. Flavonoid glycoside from the plant extract has been partially purified through chromatography and tested for efficiency against free radicals through *in vitro* antioxidant assays. The partially purified compound showed good antioxidant activity equivalent to 62.12 to 63.35 µg of rutin standard. Further cytotoxic nature of the flavonoid glycoside was also assessed against breast cancer MDA-MB-231 cell line. The tested compound has shown cancerous cell controlling ability (76.68% cell death in 24 h). The immense antioxidant and anticancer potential of flavonoid glycosides of *G. oppositifolius* could be utilized as a good source of natural, antioxidant supplement under nutraceuticals to prevent oxidative-stress-related disorders and also as therapeutic compound to cure cancer under pharmaceuticals.

Keywords: *Glinus oppositifolius,* Flavonoid glycosides, Antioxidant activity, Anticancer activity.

INTRODUCTION

Nature has developed human body to perform a healthy life when the molecules and the related mechanisms perform in an equilibrium state. Any changes in the state may attract changes in the systems of the body and may lead to short or long term diseases. The free radicals are produced in the body through a wide range of internal process and can also be produced through external stimuli. These free radicals contain subsets including reactive oxygen species (ROS) free radicals and non-ROS free radicals [1]. These molecular species are highly unstable, reactive in nature and found to be involved in certain functionalities with in the body; however, the human system is designed in a way to eliminate these free radicals on regular basis to maintain the equilibrium state through the employment of antioxidants. When there is an imbalance with the level of free radicals and antioxidants, the level of these reactive species goes high and leads to oxidative stress, which may lead to different types of cell damages and mark a starting point of many chronic diseases. There are more risk factors including lifestyle changes and different types of pollution in our environment, which is increasing the generation of the free radicals consistently inside the body.

These free radicals engage in oxidizing different essential cell molecules including carbohydrates, proteins, lipids and even DNA, which ultimately disrupts the cell function and leads to malfunctioning of the cell or its death [2]. It is always important for the body to maintain the level of these reactive species through antioxidants which are produced inside the body or acquired through the diet. When the required antioxidant level not met, the body loses its radical scavenging level and leads to onset of different types of diseases including cancer.

These highly reactive molecular species can able to damage the genomic DNA and can bring about the lack of stability in

the genome. They also involve and disturb cell signalling and can device the cell mechanism to turn on the metastatic nature of the cell and induce cancer [3]. The recent report of World Health Organization states that among one among every 6 death occurring in the world is due to cancer. This demand urges the recommendation of increasing the antioxidant intake in the day to day diet which can be achieved through dietary supplements. There are many synthetic supplements existing such as synthetic phenolic antioxidants, butylated hydroxytoluene and butylated hydroxyanisole [4].

These supplements do have their own demerits and hence the medical practitioners as well as the consumers shift their focus towards natural antioxidant compounds, which can comfortably scale up the antioxidant level of the body and helps to prevent chronic diseases including cancer. This urges finding of novel compounds from traditional medicine that can function as better antioxidant and can control these oxidative stress, reduce the risk of cancer and ensure healthy living [5]. Present work was focused on *Glinus oppositifolius* (L.). Aug. DC., an important medicinal plant belongs to the family of Molluginaceae family. The plant has been located in major parts of India, Pakistan, Philippines, Mali, Thailand, China and Nigeria. The plant has been reported in Ayurveda and found to play important role in different indigenous medicine practices. Plants possess a remarkable application in therapeutic prospective which include its antioxidant, anthelmintic [6,7], hepatoprotective [8], anti-inflammatory, analgesic activity [9] and antihyperglycemic nature [10]. Taking the traditional value of plant into account, the present study has been designed and executed to find a better compound from *G. oppositifolius* that can be used in two dimensions, controlling the free radicals and to treat the cancer condition.

EXPERIMENTAL

The leaves and stem samples of *Glinus oppositifolius* for the study has been collected from Surapet, Chennai, India and was taxonomically identified by Dr. D. Kandavel. The collected plant samples were cleaned, air dried, ground and sieved and stored in an airtight container for further use.

Extraction procedure: The plant sample was subjected to maceration-based extraction procedure under clean and good lab practices. The powdered sample was mixed with methanol in 1:10 ratio in a clean conical flask and the mouth was closed tightly. The mixture was then stirred in a laboratory stirrer under room temperature. The extraction was continued for 48 h before the sample was centrifuged and the supernatant was collected. The extracts were then dried in a desiccator. The dried powder was then stored in airtight container under refrigerated conditions.

Phytochemical analysis

Flavonoids: A diluted extract (1 mL) was added with 1% sodium hydroxide solution. The solution was observed for the appearance of yellow colour, which confirms the presence of flavonoids in the extract [11].

Tannins: Few drops of 1% lead acetate solution was added to 1 mL of extract and was observed for the formation of precipitate, which in turn indicates the presence of tannins and phenolic compounds [12].

Phenols: Few drops of 5% ferric chloride solution was added to 1 mL of extract and observed for the formation of a green-blue or violet colour which confirms the presence of compounds with phenolic hydroxyl group [13].

Free reducing sugars: 1 mL of both A and B Fehling's solution was mixed and boiled for 1 min. Then 1 mL of diluted extract was added and the complex is now kept in a boiling water bath for 10 min. It is observed for the presence of red precipitate which confirms the presence of free reducing sugars [14].

Carbohydrates: Alcoholic solution of α-naphthol (2 drops) were added to 2 mL of extract and shaken well. Then 0.5 mL of concentrated H_2SO_4 was added alongside walls off the test tube and observed for the formation of violet ring which indicates the presence of carbohydrates [15].

Saponins: An extract (2.5 mL) was heated for 5 min and then added with 1.5 mL of distilled water and vigorously shaken for 30 s. The test tube was observed for persistent froth which confirms the presence of saponins [13].

Proteins: Sodium hydroxide (10% of 1 mL solution) was added to 1 mL of extract, mixed well and then added with few drops of 1% $CuSO_4$ solution. The test solution was observed for the formation of violet or pink colour which indicates the presence of proteins [16].

Amino acids: An extract (1 mL) was taken and 2 drops of freshly prepared 0.2% ninhydrin reagent were added to the extract and heated. The appearance of pink or purple colour indicates the presence of proteins, peptides or amino acids [16].

Alkaloids: Hydrochloric acid (5 mL of 1% solution) was mixed with 2 mL of extract and heated in a water bath. 1 mL was taken from the same and few drops of Mayer's reagent was added. The mixture was observed for appearance of buff-coloured precipitate which confirms the presence of alkaloids [17].

Quinones: Few drops of concentrated hydrochloric acid were added to 1 mL of extract and the solution is observed for the formation of yellow precipitate which confirms the presence of quinones [18].

Cardiac glycosides: Glacial acetic acid (2 mL) containing 1 drop of ferric chloride solution was added to 1 mL of extract. Then 1 mL of concentrated sulphuric acid is added to the solution and observed for the appearance of brown ring which indicates the presence of cardiac glycosides in the extract [19].

Terpenes: A diluted extract (2.5 mL) was taken and 1 mL of chloroform and 1.5 mL of concentrated sulphuric acid was added. The test was observed for reddish-brown ring formation which identifies the presence of terpenes/terpenoid compounds [20].

Steroids: Screening for steroids was performed by Salkowski's Test, in which 10 mg of the extract was mixed in 1 mL of chloroform followed by addition of few drops of concentrated sulphuric acid and shaken well. The solution was allowed to stand for a while and was observed for the formation of golden yellow colour which indicates the presence of triterpenes [21].

Glycosides: An extract (2 mL) was added to 1 mL of glacial acetic acid and mixed well followed by the addition of few drops of 1% ferric chloride solution in a dropwise manner. Finally concentrated sulphuric acid was added alongside of the test tube. The test mixture was observed for the development of greenish blue colour which indicates the presence of glycosides [22,23].

Fourier transform infrared spectroscopic analysis: The extract was dissolved in methanol and the solution was analyzed using Shimadzu FTIR (IR affinity-1S). The infrared spectrum of the sample was analyzed and recorded in the region of 500 to 4000 cm^{-1}.

Gas chromatography and mass spectrophotometric analysis: The dry extract was dissolved in methanol and subjected to GC-MS analysis in Shimadzu (GC-MS-QP 2010) instrument. The gas chromatographic experiment was carried out in the specific parameters such as column oven temperature - 70 ºC, injector temperature 240 ºC, split injection mode, Split Ratio 10 and flow control in linear velocity mode. The column flow rate was maintained at 1.55 mL/min and 1 µL was used for the analysis. The carrier gas used was Helium. The mass spectrometric experiment was carried out with the specifications including 200 ºC Ion source temperature, 240 ºC interface temperature, EI (-70 eV) is the ionization level. The scan range is set from 40-1000 *m/z* and the solvent cut time is 3 min. Structure prediction is carried out using the NIST08s mass spec library. All the analysis was carried out using the software GC-MS solution ver.2.53 [24].

Separation and purification of flavonoid glycosides: The methanolic extract was subjected to silica gel (mesh size of 60-120) column chromatography for separation of the flavonoid glycosides. The fraction containing higher values of flavonoids was subjected to HPLC with C18 column (reverse phase) for further purification of the flavonoids, which was then used to test the medicinal properties [25].

Antioxidant analysis

Total antioxidant assay: Assay reagent solution with 4 mM ammonium molybdate, 28 mM sodium phosphate, 0.6 M sulphuric acid was prepared. Test sample (300 µL) was added to 3 mL of assay reagent and then the test mixture was incubated in a hot air oven at 95 ºC for 90 min. After incubation, the mixture was subjected to spectrophotometric analysis at 695 nm. Rutin was used as standard and standard curve was plotted. On comparing the test results with the standard curve, total antioxidant efficiency of the purified plant sample was calculated [26].

Ferric reducing antioxidant power (FRAP) assay: The FRAP reagent was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3 \cdot 6H_2O$ solutions in the ratio 10:1:1. To a 900 µL of FRAP reagent was added with 100 µL of test sample was added, mixed well and absorbance was measured at 593 nm. Rutin was used as standard antioxidant in the experiment and standard curve was plotted [26].

Superoxide anion scavenging activity assay: A total reagent mixture of 1.8 mL was prepared using the following composition 100 mM phosphate buffer (pH 7.4), 78 µM β-nicotinamide adenine dinucleotide (NADH), 50 µM nitro blue tetrazolium chloride (NBT) and added with 100 µL of test sample and incubated at 27 ºC for 5 min. It is then added with 100 µL of 5-methylphenazinium methosulfate (PMS) (10 µM) and incubated at 27 ºC for 5 min. Following the incubation, the absorbance was measured in a spectrophotometer at 560 nm. The percentage of scavenging was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

where, A_0 = control absorbance, A_1 = tested sample absorbance.

DPPH radical scavenging assay: A 5 mM DPPH was prepared in methanol. A 0.1mL of 1 M Tris-HCl buffer (pH 7.9) was mixed well with 1.2 mL of extract followed by the addition of 1.2 mL of DPPH solution and incubated in dark for 30 min at 27 ºC. Following the incubation, the absorbance was measured at 517 nm. The procedure was repeated for different concentrations of the extract [26]. Rutin was used as the standard antioxidant compound. The percentage of scavenging was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Anticancer activity: The anticancer effect of the sample was tested against breast cancer MDA-MB-231 cell line by MTT assay. The breast cancer cells at a concentration of 10^6 cells/well were seeded in micro plates and incubated at 37 ºC for 48 h in 5% CO_2 incubator and allowed to grow 70-80% confluence. After incubation, old medium was replaced with fresh medium and added with different increasing concentration of the partially purified flavonoid glycoside (25, 50, 75, 100, 125 µg/mL). The plate was again incubated for 24 h and following the incubation the cells were washed with phosphate buffer saline (PBS, pH-7.4) and 20 µL of MTT solution (5 mg/mL in PBS) was added to each well. The plates were then kept at 37 ºC in the dark for 2 h. After incubation 100 µL DMSO was added and the absorbance was read spectrophotometrically at 570 nm [27]. The cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100$$

RESULTS AND DISCUSSION

The plant kingdom is a treasure hive of potential medicines and herbal products. Due to presence of various useful phytochemicals, plants are considered for potential source of variety of therapeutic applications. The present study was carried out to identify a potential compounds from the *Glinus oppositifolius* (Fig. 1), which can act as nutraceutical antioxidant supplement to prevent oxidative stress and as a cancer cure medicine. In the present study, the preliminary phytochemical screening of methanolic extract of *G. oppositifolius* revealed the presence of flavonoids, tannins, polyphenols, carbohydrates, amino acids, alkaloids, glycosides, cardiac glycosides, terpenes and steroids were found to be present in abundance. The obtained

Fig. 1. Leaf and stem parts of *G. oppositifolius*

phytochemical profiles (Table-1) was promisingly affirms that the plant could possess one or more valuable lead compound with therapeutic efficacy.

TABLE-1
PHYTOCHEMICAL CONSTITUENTS OF METHANOL EXTRACT OF *G. oppositifolius*

Phytochemicals	Observation
Flavonoids	Very highly present
Tannins	Very highly present
Phenols	Very highly present
Free reducing sugars	Present
Carbohydrates	Very highly present
Saponins	Highly present
Proteins	Highly present
Amino acids	Very highly present
Alkaloids	Very highly present
Glycosides	Very highly present
Cardiac glycosides	Very highly present
Terpenes	Very highly present
Steroids	Very highly present

FTIR studies: FTIR analysis identified several functional compounds in the methanolic extract of *G. oppositifolius*. The vibrations observed at 3361.93 cm^{-1} is attributed to O-H hydroxy group stretching vibration of polyhydroxy compound of methanolic extract. The peak observed at 2941.44 cm^{-1} is attributed to the C-H stretching vibration of alkenes. Similarly, Kamble I Gaikwad reported that methanolic leaf extract of *Embelia ribes* exhibited the peaks at 3344.93 and 2936.09 cm^{-1} confirms the presence of alcohol, amines, amides and alkanes, respectively [27]. The peaks observed at 2592.33 and 2519.03 cm^{-1} are attributed to O-H stretching vibration of carboxylic acids. Santhi & Sengottuvel [28] revealed that the absorption spectra of methanolic leaf extract of *Moringa concanensis* has shown the peat peak at 2524.07 cm^{-1} assigned for O-H stretching in the carboxylic acids. The elevations observed at 2362.8, 2343.51 and 2227.78 cm^{-1} are attributed to C-C stretching vibration of terminal alkynes. Like that the C≡C stretching was found to be alkynes present due to the appearance of absorption peak at 2226.40 cm^{-1} [28]. The peaks at 1436.97 and 1406.11 cm^{-1} are attributed to O-H bend present in phenol or *tertiary* alcohol. Chikwe & Arthur [29] reported that the peak at 1400 cm^{-1} assigned to the O-H stretching vibration, O-H bending vibration and O-H bending medium vibration respectively shows that some alcoholic compounds are present in the leaf extracts. The vibrations observed at 1031.92 and 952.84 cm^{-1} are attributed to phosphate ion group stretching vibration of phosphate compound. The peak observed at 669.3 cm^{-1} is attributed to C-Br stretching vibration of aliphatic bromo compounds. Similarly, the peak at 1045.23, 879.381 and 635.43 cm^{-1} indicates the functional group corresponding to aliphatic amines, primary or secondary amines, *p*-substituted alcohols or phenols, alkynes, alkyl halide and disulphide [27].

GC-MS analysis: GC-MS analysis of methanolic extract of *G. oppositifolius* revealed the presence of several phytochemicals, which are shown in Fig. 2. GC-MS analysis revealed the presence of methyl 2-O,3-O-dimethyl-α-D-xylofuranoside, 4,4-dimethyl-2-propenyl cyclopentanone, *n*-hexadecanoic acid, phytol, 9,12,15-octadecatrienoic acid, 9,12-octadecadien-1-ol, 2-tridecanone, 9-eicosyne, 9-octadecenal, hexadecanoic acid 2-hydroxy-1-(hydroxymethyl)ethyl ester, 2(1*H*)-naphthalenone, octahydro-4a-methyl-7-(1-methylethyl)-; (4aα, 7β, 8aβ), *trans* 13-docosenamide, squalene, γ-tocopherol, 5-acetamido-4,7-dioxo-4,7-dihydrobenzofurazan, (*E*)-2-bromobutyl oxy chalcone, arsenous acid, *tris*(trimethylsilyl) ester and stigmasta-7,25-dien-3β-ol.

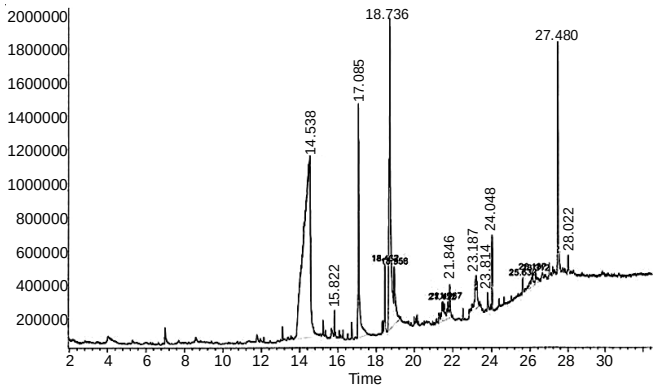

Fig. 2. GC-MS spectrum of *G. oppositifolius*

Antioxidant activity: The antioxidant activities in the partially purified flavonoid glycosides of *G. oppositifolius* are presented in Fig. 3. Total antioxidant (TA) capacity of the test compound was measured by phosphomolybdenum method where the ability of the compound to reduce Mo(VI) to Mo(V) was tested. Test compound (100 µg) was found to be containing reducing power which is equivalent to 63.35 µg of the standard rutin.

The flavonoid glycosides were subjected to ferric reducing power assay (FRAP) where the compound's ability to reduce Fe^{3+} to Fe^{2+} in the presence of tripyridyltriazine (TPTZ) was tested. Conversion of ferric to ferrous reduction is proportional

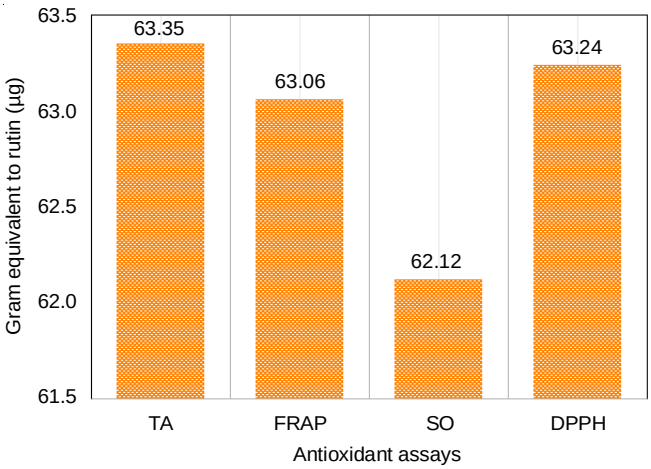

Fig. 3. Antioxidant activity of flavonoid glycosides of *G. oppositifolius*

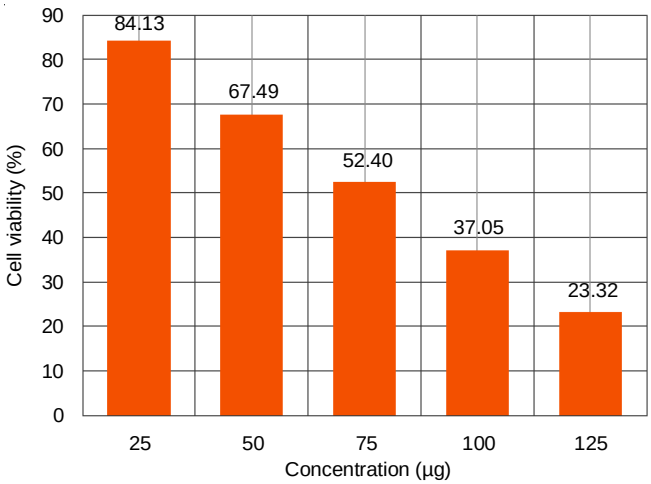

Fig. 5. MDA-MB-231 cell viability against different concentration of flavonoid glycoside

to antioxidant ability of the molecules. Test compound (100 µg) has shown reducing power which is equivalent to 63.06 µg of rutin standard. The isolated flavonoid glycoside was tested for its ability to scavenge the superoxide anion (SO), ROS free radical. Test compound (100 µg) exhibited a scavenging ability that is equivalent to 62.12 µg of rutin standard. Through the DPPH assay, it was found that 100 µg of flavonoid glycosides of *G. oppositifolius* contains antioxidant power which is equivalent to 63.24 µg of rutin standard.

Anticancer activity: In present study, the cytotoxic activity of flavonoid glycosides of *G. oppositifolius* on breast cancer MDA-MB-231 cell line was examined through MTT assay. The flavonoid glycoside was found potent to exhibit cytotoxic extracts against MDA-MB-231 cancer cell lines (Fig. 4). Treatment with flavonoid glycosides of *G. oppositifolius* has reduced the viability of MDA-MB-231 cells in a dose dependent manner after 24 h of exposure (Fig. 5). The best reduction of percentage of cell viability (76.88%) has been observed against MDA-MB-231 cell lines at maximum concentration 125 µg/mL. The IC_{50} value of flavonoid glycosides of *G. oppositifolius* against MDA-MB-231 cell lines was found to be 79.74 µg/mL (Fig. 6).

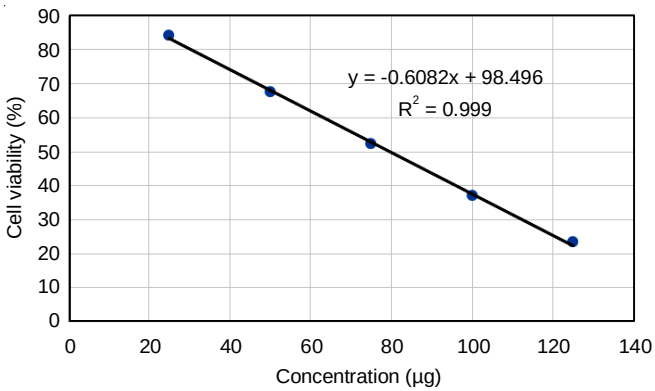

Fig. 6. IC_{50} analysis of flavonoid glycoside against MDA-MB-231 cell line

Conclusion

In present study, the methanolic extract of aerial parts of *Glinus oppositifolius* showed higher concentrations of amino acids, flavonoids, polyphenols, carbohydrates, alkaloids, glycosides, cardiac glycosides, terpenes, steroids and tannins. The FTIR analysis indicates the functional groups of aromatic

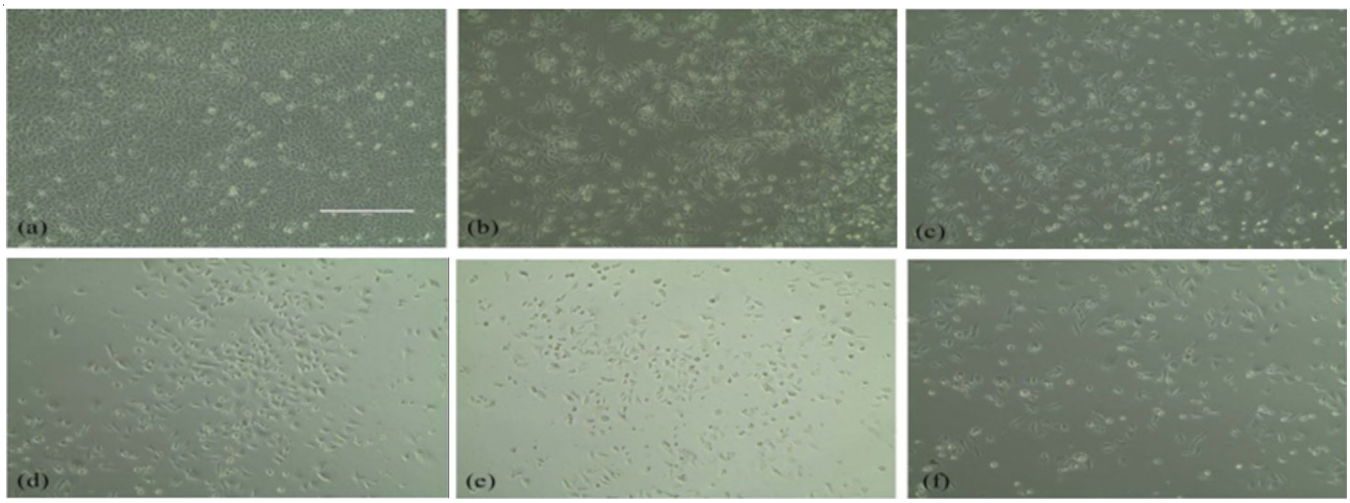

Fig. 4. MDA-MB-231 cell line growth in different concentration of flavonoid glycoside [(a) control, (b) 25 µg/mL, (c) 50 µg/mL, (d) 75 µg/mL, (e) 100 µg/mL and (f) 125 µg/mL]

compounds, carboxylic acids, alkanes and alkyls and GC-MS analysis reflects the presence of a range of therapeutic organic compounds, especially phenolic/flavonoid derivatives and carbohydrate based moieties. The partially purified flavonoid glycosides of *G. oppositifolius* have shown better antioxidant activity in the assays performed. The compound also shows an appreciable cytotoxic effect on the tested breast cancer MDA-MB-231 cell line, which proven its anticancer potential. It is clearly stated that *G. oppositifolius* displayed potent anti-oxidant activity on various assays and also exhibit anticancer activity on breast cancer cells.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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