Evaluation of Cytotoxic Effects of Methanolic Extract and Fractions of *Mirabilis jalapa* (L.) Leaf

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Authors’ contributions

This work was carried out in collaboration among all authors. The work was designed by authors SOA and BAA. The cytological aspect was done by authors SOA, ANO, AG and BAA did the extraction, *Allium cepa* bioassay and phytochemical screening. Authors BAA and JTA carried out the Brine Shrimp bioassay. All the authors read and approved the final manuscript.

ABSTRACT

This study examined the potential cytotoxicity of *Mirabilis jalapa* L. methanolic crude leaf extract and its fractions against brine shrimp nauplii (*Artemia salina* L.) and *Allium cepa* L. roots. The leaf extraction was done according to standard technique and crude extract was partitioned using n-hexane, water, ethyl acetate and butanol to obtain their respective fractions. *Allium cepa* root growth inhibition of *M. jalapa* methanolic crude extract and fractions were evaluated as well as brine shrimp lethality of the fractions based on standard methods. Also, phytochemical screening of the methanolic crude leaf extract was carried out according to standard methods. The result showed that *M. jalapa* methanolic crude leaf extract caused a significant reduction in cell mitotic index (32.96%) compared with the control (52.13%). The butanol fraction produced the highest mitotic inhibitory activity on *A. cepa* cell division at 0.3 mg/ml. Moreover, the butanol fraction produced the highest percentage lethality (LC50 1.45 μg/ml) against brine shrimp nauplii. There was a strong correlation between brine shrimp lethality and mitotic cell inhibition with butanol fraction as the most potent in both models. The methanolic leaf crude extract tested positive for alkaloids, cardiac glycosides, flavonoids, saponins, steroids, tanins and triterpenes. The methanolic crude extract of...
M. jalapa leaf and its fractions exhibited effective cytotoxic effect on A. cepa and brine shrimps. Butanol fraction, with the most cytotoxic activity among the tested extracts, demonstrates a promising source for novel anticancer agents.

Keywords: Mirabilis jalapa; cytotoxicity; brine shrimps; Allium cepa; mitotic index.

1. INTRODUCTION
Mirabilis jalapa L. belongs to a small family, Nyctaginaceae consisting majorly New world tropical and subtropical species [1]. It is a perennial weedy plant found in disturbed areas, meadows and fields. It possesses different flower colors which include yellow, white, magenta and pink [2, 3]. Mirabilis jalapa is a well-known ornamental plant that is cultivated throughout the world for its beautiful and pleasing fragrance flowers [2,3]. It is popularly known as 4 O’ clock plant and it is called “Tonaposo” among Yoruba people of Nigeria [4]. The leaves are ovate shape, green, and bitter with a characteristic odour [5,6]. A chromosome number of 2n = 54 [7] and 2n= 58[8] was reported in M. jalapa.

Different parts of M. jalapa have been studied by many researchers. Phytochemical constituents such as phenols, alkaloids, glycosides, flavonoids, saponins, steroids, tanins, triterpenes and anthraquinones were reported to be present in different parts of M. jalapa [6]. A wide range of biological activities of the plant extracts has also been documented. These include anti-inflammatory, anti-bacterial, anti-microbial, anti-oxidant, anti-cancer, anti-nociceptive, anti-spasmolytic, anti-viral, anti-fungal, anti-diabetic, anti-helminthic, anti-malarial anti-stress and immune stimulating activities [3, 5, 9, 10].

Mirabilis jalapa is used in the treatment of various ailments such as dropsy (inflammatory oedema), fever, menstrual disorders, constipation, and diarrhea. In addition, it is used as aphrodisiac, diuretic and laxative (cleanser) [2,3]. In Nigeria, the leaves are consumed as memory enhancer [4]. Moreover, an edible red dye for coloring food was obtained from M. jalapa flower [2]. Its leaves have also been reported to be cooked as food during an emergency case only [3]. In spite of these acclaimed uses in ethno medicine, the whole plant of M. jalapa was reported to be poisonous [11]. Hence, this study was undertaken to investigate the potential toxic effects of M. jalapa using Allium cepa and Brine Shrimp models.

Allium cepa assay is an important in vivo test used by researchers to evaluate the toxicity of any chemical agent or substance. The roots are grown in direct contact with the test material enabling possible damage to DNA of humans to be predicted [12,13,14]. Additionally, brine shrimp lethality assay (BSLA) is a simple and economical bioassay used globally for testing the efficacy of chemical agents and plant extracts for presence of toxic principle(s) or effects of test sample on acute overdose [15,16]. Such lethality in a simple organism like Artemia salina. (Brine shrimp nauplii) has been employed by various investigators as suitable tool for screening chemical agents for toxic effects. In BSLA, the organism is directly exposed to the test sample, and after 24 hours, the number of nauplii that survive is counted and the percentage mortality is determined using an appropriate equation [14, 17].

2. MATERIALS AND METHODS

2.1 Plant Sample Collection
Fresh leaf sample of Mirabilis jalapa was collected from Ido Osun, Ile-Ife, Osun State, Nigeria (7°27” 7.49N, 4° 34” 4.56E) and authenticated at the IFE Herbarium, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. A voucher specimen with voucher number IFE-17999 was deposited at IFE Herbarium (Fig 1).

2.2 Preparation of M. jalapa Leaf Extract
The leaves were air dried for three weeks and milled into powder. The powdered sample (15 g) was extracted with 80% (v/v) methanol for 48 h, and filtered with No 1 Whatman filter paper. The residue was soaked in freshly prepared 80% (v/v) methanol and the process was repeated till a clear solution was obtained. The filtrates were pooled and concentrated in vacuo on a rotary evaporator at 40 °C to yield the methanolic crude extract.

2.3 Partitioning of Crude Methanolic Extract of M. jalapa Leaf
The crude methanolic leaf extract of M. jalapa was dissolved in distilled H₂O and successively
partitioned with n-hexane (n-Hex), ethyl acetate (EtOAc), butanol (BuOH) and water (Aqueous) [13]. The solvent fractions were concentrated in vacuo on rotary evaporator to produce n-hexane, ethyl acetate, butanol and aqueous fractions respectively.

2.4 Allium cepa Bioassay

Methanolic crude extract and fractions (n-hexane, ethyl acetate, butanol and aqueous) of M. jalapa leaf were evaluated for root growth inhibitory potential using Allium cepa model [13]. Fresh onion bulbs were purchased from Ile-Ife local market and sundried for three weeks. The rooting was initiated in distilled water for 48 hours. The sprouted bulbs were planted separately in 0.0, 0.10, 0.20, 0.30 and 0.40 mg/ml of the extract and fractions (n-hexane, ethyl acetate, butanol and aqueous) of M. jalapa for 24 hours while the sprouted bulbs in distilled water served as the control. Thereafter, the roots were harvested and fixed in acetic acid/ethanol (1:3 v/v) for 24 hours and stored in refrigerator for further analysis. Five roots from each bulb were measured and the mean root length was calculated.

2.5 Cytological Examination of the Allium cepa Root Tips

The fixed roots were hydrolyzed in HCl (18% v/v) for 10 minutes and the root tips were squashed and stained with FLP-orcein for 15 minutes [14]. A total of twenty-five fields was viewed at random from five slides; the total number of cells undergoing mitotic division and the total number of non-dividing cells were counted. Photomicrographs of the cells were taken using ACU-scope trinocular microscope (ACCU-scope 33001 LED trinocular microscope fitted with 3.2 MP (MOS digital camera). The mitotic index was calculated as:

\[
\text{Mitotic Index} = \frac{\text{Number of dividing cells} \times \text{Total number of cells}}{100}
\]

2.6 Brine Shrimp Lethality Bioassay

The various fractions of M. jalapa were assessed for lethality to brine shrimp larvae following standard method [17]. The eggs of brine shrimp were hatched using brine shrimp eggs in a hatching chamber (1L) filled with sterile artificial seawater under constant aeration for 48 hours. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Each fraction was screened separately at 0, 100 and 1000 μg/ml in 10 ml sea water solutions with 1% DMSO (v/v) as vehicle that dissolved the test sample. With the aid of a Pasteur pipette, 10 living nauplii were transferred to each test concentration, 1% DMSO or sea water (negative control) as well asDiclofenac solution (positive control) in vials maintained at room temperature for 24 hours under the light. After 24 hours, the number of nauplii survived in each vial was counted with the help of magnifying glass. The assay was performed in triplicate. The lethal concentration that produced 50% mortality after 24 hours of exposure (LC50) was determined using the probit method. The LC50 values greater than 1000 μg/ml for plant extracts were considered inactive [17].

2.7 Phytochemical Screening of M. jalapa Methanolic Crude Leaf Extract

The phytochemical screening of the methanolic leaf crude extract of M. jalapa was carried out according to standard methods [18,19].

2.8 Statistical Analysis

Data obtained were subjected to statistical analysis using One-Way Analysis of Variance. Data was considered significant at p<0.05. Data were expressed as mean with standard error in Brine Shrimp assay. Duncan’s multiple range tests was employed to determine the significant difference among the mitotic parameters for different concentrations in A. cepa assay.

3. RESULTS AND DISCUSSION

3.1 Effect of Methanolic Crude Leaf Extract of M. jalapa on Allium cepa Roots and Mitotic Index

At higher concentrations of the plant extract, the Allium cepa roots showed little or no further growth after 24 hours of exposure. Roots placed in control (distilled water) and 0.1 mg/ml appeared healthier, longer and bigger compared to the roots exposed to higher concentrations. The highest root growth inhibition (38.09%) was recorded at 0.2 mg/ml; while the least inhibition (13.80%) was obtained at 0.1 mg/ml. Also, there was a significant reduction in the mitotic index (32.96%) of the roots treated with different concentrations of M. jalapa when compared with the control (52.13%). The effects of the methanolic crude extract on A. cepa root growth...
and mitotic index were not dose dependent and no chromosomal aberration was observed (Table 1; Fig 2).

Toxic effects of pollutants, drugs or herbal preparations have been investigated by analyzing macroscopic parameters (such as root number and root growth) while genotoxicity and cytotoxicity were evaluated by investigating the cytological parameters [20]. Root growth inhibition over 45% indicates presence of toxicants with sub-lethal effects on the test plants [21]. However, the highest root growth inhibition recorded in the methanolic crude extract was 38.09% which showed that the tested crude extract of *M. jalapa* did not possess any toxicant with sub-lethal effect. The percentage root growth inhibition was observed to be correlated with the mitotic activity of the cells. Nevertheless, the observed wilted and stunted growth in the immersed roots in the methanolic crude extract and fractions of *M. jalapa* at concentrations higher than 0.1 mg/ml were indication of cytotoxicity. Stunting and wilting roots are considered as signs for anti-mitotic activities [22]. Significant decrease in mitotic index suggests mito-suppressive action of the compound, indicating that *M. jalapa* extract interfered with the normal sequence of cell cycle which consequently reduced the number of dividing cells [23].

![Fig. 1. Habit of *Mirabilis jalapa*](image)

**Table 1. Effect of the *M. jalapa* methanolic leaf crude extract on root growth and mitotic index**

| Conc. (mg/ml) | Root length Mean (cm) | Growth inhibition (%) | Mitotic Index (%) |
|---------------|-----------------------|-----------------------|-------------------|
| 0             | 2.10±0.16^a           | 0.00                  | 52.13^a           |
| 0.1           | 1.81±0.24^b           | 13.80^d              | 42.73^b           |
| 0.2           | 1.30±0.14^e           | 38.09^e              | 32.96^d           |
| 0.3           | 1.57±0.21^d           | 25.24^c              | 38.37^e           |
| 0.4           | 1.78±0.21^c           | 15.23^b              | 40.64^d           |

Values with different letters are significantly different from each other at p≤0.05
Fig. 2. Stages of Cell division in *A. cepa* at different concentrations

A. Anaphase (Control); B. Anaphase (0.1 mg/ml); C. Metaphase (0.2 mg/ml); D. Metaphase (0.3 mg/ml); E. Anaphase (0.4 mg/ml)

### 3.2 Effect of *M. jalapa* Leaf Fractions on *Allium cepa* Root Growth and Mitotic Index

A non-significant decrease in root length was observed at different concentrations of *M. jalapa* fractions (Table 2) except for aqueous fraction which enhanced root growth at 0.2 and 0.3 mg/ml. The butanol leaf fraction significantly inhibited the *A. cepa* root growth across all the concentrations tested. The n-hexane, EtOAc and BuOH fractions inhibited the *Allium cepa* root growth only at lower concentrations. This suggested that the butanol fraction contained significant amounts of mito-suppressive constituents which interfered with *Allium cepa* cell division. The reduction in mitotic activity could also be due to inhibition of DNA synthesis which is one of the major prerequisites for cell division [24]. In biological system, there are some surveillance mechanisms that have been in place to detect different DNA damage which act in many ways to maintain genome integrity; one of the ways is the blockage of DNA replication to slow or stop cell cycle progression [25]. Consequently, there will be reduction in cell mitotic activity. In addition, DNA breakage at the replication site is being stimulated by inhibition of DNA replication resulting in cytotoxicity [26].

| Sample (mg/ml) | 0.1  | 0.2  | 0.3  | 0.4  |
|---------------|------|------|------|------|
| Root length Mean (cm) |      |      |      |      |
| Control       | 3.54±0.17 a |      |      | 3.56±0.25 a |
| n-Hex Fraction| 3.30±0.15 a | 3.28±0.16 a | 2.32±0.25 a |      |
| EtOAc Fraction| 3.20±0.28 a | 3.38±0.44 a | 3.72±0.27 a | 3.92±0.26 a |
| BuOH Fraction | 1.78±0.14 b | 2.58±0.23 b | 2.88±0.33 b | 3.32±0.18 a |
| Aq Fraction   | 2.50±0.14 b | 4.20±0.29 a | 4.34±0.61 a | 3.08±0.24 a |

Values with different letters are significantly different from each other at p≤0.05
More so, the decrease in the mitotic index might be caused by the decreasing ATP level and pressure from the functioning of the energy production center [27]. Furthermore, inhibition of DNA polymerase, which is necessary for the synthesis of DNA precursors as well as other enzymes more directly involved with spindle production, assembly or orientation, could also account for the reported antimitotic effects [28]. The BuOH leaf fraction demonstrated the highest level of potency (Table 3), followed by aqueous fraction while ethyl acetate fraction showed the least potency at inhibiting A. cepa cell division. The ethyl acetate fraction stimulated cell mitosis at 0.2 mg/ml but strongly inhibited cell division at higher concentrations (Table 3) and caused chromosomal aberrations (clumped metaphase), cell elongation and cell degeneration (Fig. 3). At higher concentration in n-hexane fraction, cell degeneration was observed in addition to normal cells. The absence of chromosomal aberrations in the cells treated with the methanolic crude extract indicated that the extract elicits no genotoxic effect at the concentration tested. It should be noted that crude therapeutic products are less toxic which was said to be due to synergistic interaction of the active ingredients in them [29]. However, cell and chromosomal abnormalities were observed in some cells treated with the fractions of the leaf extracts (BuOH and EtOAc). In this current study, M. jalapa leaf extract fractions showed both cytoxic and genotoxic effects; indicating the toxicity of the plant extract. The cytoxic and genotoxic activities of a plant extract are very important in evaluating cytotoxicity and screening of a prosperous pharmacological important drug [29,30].

3.3 Effect of M. jalapa Leaf Fractions on Brine Shrimp Lethality

The effect of the leaf fractions of M. jalapa on shrimp nauplii (Table 4) was concentration dependent. The highest percentage mortality was recorded after 24 hours at 1000 μg/ml. All the shrimp nauplii survived in the control medium (sea water). The butanol fraction produced the highest percentage lethality on brine shrimp nauplii (LC₅₀ 1.45 μg/ml) and was considered the most potent fraction. This was followed by the ethyl acetate fraction (LC₅₀ 48.33 μg/ml) while n-hexane fraction as well as aqueous fraction showed low level of potency and were considered inactive. There was a strong correlation between brine shrimp lethality and mitotic cell inhibition with butanol fraction as the most potent in both models.

In brine shrimp assay, LC₅₀<1.0 μg/ml is considered highly toxic; LC₅₀ 1.0-10.0 μg/ml is toxic; LC₅₀ 10–30 μg/ml is moderately toxic; LC₅₀ 30 – 100 μg/ml is mildly toxic, and > 100μg/ml is non-toxic [31]. Therefore, n-hexane and aqueous fractions, DMSO and diclofenac which had LC₅₀ above 100 μg/ml were practically non-toxic. Nevertheless, butanol fraction with LC₅₀ value of 1.45 μg/ml was toxic while ethyl acetate fraction (LC₅₀ - 48.33 μg/ml) was mildly toxic. Plant extracts with LC₅₀< 20 μg/ml have a tendency to yield anticancer compounds [31]. Moreover, The brine shrimp lethality bioassay has been shown to have a positive correlation with cytotoxic activity in some human solid tumors which has given way to the discovery of novel active antitumoral agents [32]. Therefore, the butanol leaf fraction of M. jalapa has the potential to offer new lead molecules for cancer therapy. The LC₅₀ cutoff point has also been extensively suggested elsewhere [32, 33]. An anti-cancer compound Englerin A was isolated from the extract of Phyllanthus engleri which was shown to have selective anti-cancer property against kidney cancer cells [33]. Such findings provide further corroborative evidence on the potential of brine shrimp test to predict the presence of anti-cancer compounds in plant extracts. Cytotoxic action of a drug was said to have a relationship with the disturbance of important mechanisms that are associated with cell growth, mitotic activity, differentiation and function [34] as observed in this study. Therefore, the results from this present study, suggested the presence of anticancer bioactive components in the butanol fraction of M. jalapa leaf.

3.4 Phytochemical Constituents

The methanolic crude leaf extract of M. jalapa tested positive for alkaloids, cardiac glycosides, flavonoids, saponins, steroids, tannins and triterpenes (Table 5). Qualitative analysis of the result showed that cardiac glycosides, steroids and triterpenes were more abundant than the rest of phytochemicals screened. The presence of alkaloids, glycosides, flavonoids, tannins, steroids and triterpenes in the plant studied correlated with other reports.[6,9,10] Phytochemicals are biologically active, naturally occurring chemical compounds found in plants. Phytochemicals have been reported to be responsible for various plant biological activities and provide health benefits for humans as
medicinal ingredients and nutrients [35,36]. Recently, attention is being given to plant secondary metabolites over the synthetic chemicals because these plant products have been shown to be useful pharmaceutically [37]. More so, the antibacterial and antioxidant activities of M. jalapa have been attributed to the presence of phytochemicals in the plant [38].

Table 3. Effect of the methanolic leaf fractions of M. jalapa on mitotic index of A. cepa

| Conc. (mg/ml) | Control | EtOAc Fraction | n-hexane fraction | BuOH fraction | Aqueous fraction |
|---------------|---------|----------------|-------------------|---------------|-----------------|
|               | 37.59   |                |                   |               |                 |
| 0.1           | 33.61<sup>b</sup> | 21.03<sup>a</sup> | 2.70<sup>a</sup>  | 21.32<sup>a</sup> |
| 0.2           | 43.06<sup>a</sup> | 25.19<sup>a</sup> | 0.96<sup>b</sup>  | 10.11<sup>b</sup> |
| 0.3           | 30.35<sup>b</sup> | 17.59<sup>b</sup> | 0.57<sup>b</sup>  | 4.19<sup>c</sup>  |
| 0.4           | 11.48<sup>c</sup> | 21.00<sup>a</sup> | 1.45<sup>a</sup>  | 7.80<sup>c</sup>  |

Values with different letters are significantly different from each other at p≤0.05

Fig. 3. Stages of cell division in A. cepa at different concentrations
A. Metaphase (blue arrow) and Prophase (red arrow) (DMSO); B. Anaphase (DMSO); C. Prophase (blue arrow) and Early Telophase (red arrow) (EtOAc 0.1 mg/ml); D. Anaphase (EtOAc 0.1 mg/ml); E. Clumped Metaphase (EtOAc 0.2 mg/ml); F. Elongated cells (EtOAc 0.2 mg/ml); G. Degenerated cells (EtOAc 0.4 mg/ml); H. Elongated cells (BuOH 0.1 mg/ml); I. Different stages of cell division (N-Hex 0.4 mg/ml)
### Table 4. Percentage mortality of shrimp nauplii Treated with M. jalapa leaf fractions

| Test Sample | CONC. (µg/ml) | At 0 hour Mortality (%) | After 6 hours Mortality (%) | After 12 hours Mortality (%) | After 18 hours Mortality (%) | After 24 hours Mortality (%) | LC₅₀ (µg/ml) |
|-------------|---------------|-------------------------|-----------------------------|-----------------------------|----------------------------|----------------------------|-------------|
| BuOH Fraction | 10            | 0                       | 5                           | 20                          | 45                         | 85                         | 1.45        |
| 100         | 0             | 10                      | 15                          | 25                          | 55                         | 95                         |
| 1000        | 0             | 5                       | 30                          | 65                          | 95                         |
| EtOAc Fraction | 10            | 0                       | 0                           | 5                           | 30                         | 70                         | 48.33       |
| 100         | 0             | 10                      | 15                          | 25                          | 40                         | 75                         |
| 1000        | 0             | 20                      | 35                          | 65                          | 100                        |
| Aqeous Fraction | 10            | 0                       | 10                          | 20                          | 40                         | 65                         | 3386.28     |
| 100         | 0             | 10                      | 15                          | 25                          | 40                         | 65                         |
| 1000        | 0             | 5                       | 35                          | 65                          | 100                        |
| n-Hex Fraction | 10            | 0                       | 15                          | 25                          | 45                         | 75                         | 1172.85     |
| 100         | 0             | 5                       | 35                          | 65                          | 75                         |
| 1000        | 0             | 10                      | 20                          | 45                          | 75                         |
| DMSO (vehicle) | 10            | 0                       | 0                           | 5                           | 15                         | 95                         | 2648.13     |
| 100         | 0             | 0                       | 0                           | 10                          | 30                         |
| 1000        | 0             | 5                       | 0                           | 15                          | 50                         |
| Diclofenac (control drug) | 10            | 0                       | 10                          | 5                           | 20                         | 85                         | 1002.08     |
| 100         | 0             | 0                       | 35                          | 65                          | 85                         |
| 1000        | 0             | 35                      | 40                          | 80                          | 100                        |
| Sea water   | 10            | 0                       | 0                           | 0                           | 0                          |
| 100         | 0             | 0                       | 0                           | 0                           |
| 1000        | 0             | 0                       | 0                           | 0                           |

### Table 5. Phytochemical constituents of *M. jalapa* crude extract

| Phytochemical Constituents | Presence |
|----------------------------|----------|
| Alkaloids                  | +        |
| Cardiac Glycosides         | ++       |
| Flavonoids                 | +        |
| Saponins                   | +        |
| Steroids                   | ++       |
| Tannins                    | +        |
| Triterpenes                | ++       |

*+ present; ++ moderately present*
4. CONCLUSION

_Mirabilis jalapa_ leaf extract exhibits cytotoxic, genotoxic and lethal effects on _A. cepa_ and brine shrimps nauplii. However, the butanol leaf fraction LC50 value suggested that _M. jalapa_ leaf is a promising source for novel anticancer agents. Therefore, further studies on isolation of potential antitumour and cytotoxic agents from the plant are strongly recommended.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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