

Prooxidant Effect of the Crude Ethanolic Leaf Extract of *Ficus odorata* Blanco Merr. *in vitro*: It's Medical Significance

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Abstract—Alongside with antioxidant, pro-oxidant activity is also observed in phytochemical compounds. In the study, *Ficus odorata*, an endemic medicinal plant in the Philippines, was screened for the potential medical application of its pro-oxidant activity.

Phytochemical screening revealed the presence of terpenes, glycosides and phenolic acids. The crude extract was found to contain low gallic acid and quercetin equivalence. The TLC chromatogram of the crude extract showed that none of the 11 spots obtained has antioxidant activity nor correspond to gallic acid and quercetin standards. Experiments showed that the crude extract has stimulatory activity towards DPPH radicals, hydrogen peroxide, hydroxyl radicals, superoxide anions and nitric oxide. Moreover, the extract exhibited a low ferric reducing power.

The prooxidant activity was evident in the crude ethanolic leaf extract of *F. odorata*, which may provide a better understanding of the plant's pharmacological importance in the prevention of diseases.

Keywords—*Ficus odorata* Blanco, Free Radicals, Oxidative Stress, Prooxidant, Antioxidant.

I. INTRODUCTION

WHILE some of food substances, at low concentration, have antioxidant activity, it is of particular interest that they may also possess a pro-oxidant activity depending on the cellular environment and conditions [1]. Among of the well-known and commercially available natural antioxidants are vitamins C and E, carotenoids and some polyphenolic compounds [2] and [3]. They are believed to elicit specific functions inside the body and contribute in maintaining good health. However, there are current notions that some of these exogenous antioxidants have additional role as a pro-oxidant especially when administered at high doses. Bouayed and Bohn [4] discussed that the balance between the beneficial and deleterious activity of the exogenous antioxidants may have been related to the type, dosage and matrix of these compounds and is also dependent on the redox potential of these molecules and the inorganic chemistry of the cell [2] and [5].

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Pro-oxidant actions are considered to have a more specific preference against certain cellular targets and were observed to play an important role in the prevention of tumor progression [6]. One mechanism that explains the anticancer property of pro-oxidants involved DNA degradation with the aid of transition metal ions such as copper (Cu^{2+}) and iron (Fe^{2+}) [7] and [8].

Among the most studied phytochemicals, polyphenolic compounds were reported to elicit such mechanism as shown in some studies [7]-[9]. Aside from the apoptotic function that is related to the pro-oxidant activity exhibited by some plants, other biologically important reactions in the body also involve the presence of reactive oxygen species (ROS). Normally, the body produces ROS and reactive nitrogen species (RNS) as by-products of metabolic reactions that occurs within the cells. For instance, superoxide and hydrogen peroxide are produced by phagocytic cells in order to kill invading microorganisms. RNS such as nitric oxide is also biologically present and is usually connected to cardiovascular diseases, aging, and regulation of brain functions.

Among the 150 endemic *Ficus* species in the Philippines, *Ficus odorata* is one of the medicinal plants that are less studied in terms of its pharmacological functions. The plant is locally known as “pakiling” or “isis” in the Tagalong region and is commonly used in the treatment of allergy, asthma, diarrhea, diabetes, tumor and cancer [10]. It is widely cultivated in Luzon such as Tayabas, Zambales, Pampanga, Bataan, and Laguna. The plant is classified as a small tree, reaching nine meters high with young hispidous, greenish branchlets. Leaves are simple, alternate, oblong or broadly ovate and rounded, 16-20cm long 6-8cm wide, acuminate at apex, cordately inequilateral, margins toothed, lateral veins 6-8 pairs, prominent underneath, base penta to octa-veined, venation semicraspedodromous. The dried leaves are fragrant. Young branches are greenish, pubescent, petiole 1.0-1.2cm long, cylindrical and stipule 0.5-1.0cm long, deciduous [11] and [12].

There are studies that described the antioxidant activity of some *Ficus* species such as *Ficus pseudopalma* Blanco, *Ficus bengalensis* L., *Ficus racemosa* L., and *Ficus glomerata* [13]-[15]. However, the crude ethanolic leaf extract of *F. pseudopalma* was shown to stimulate hydroxyl radicals and superoxide anions [13]. The anticancer activity of *F. pseudopalma* was also discussed in the study conducted by

Bueno et al. [16] and was comparable to the activity of curcumin.

In view of this, since there are scant data regarding the pharmacological activity of *F. odorata*, the study was conducted to understand better the possible biological functions of the plant and the mechanism by which it can elicit these functions.

II. MATERIALS AND METHODS

A. Chemicals and Reagents

Analytical grade solvents were used in all of the experiments and were purchased from RCI Labscan Limited, Bangkok, Thailand. All standards and reagents used in the antioxidant assays were purchased from Sigma-Aldrich Co. St. Louis, Germany.

B. Plant Preparation and Extraction

Ficus odorata leaves was authenticated on the Botany Section of the Philippine National Museum. The plant extraction was performed based on the discussed procedure [13]. Briefly, one kilogram of ground air-dried leaves was soaked with 10L 95% ethanol in a percolator for four days. The ethanolic extract was collected every 24 hours and was replaced with succeeding batches of ethanol. The filtrates for each collection were concentrated through a rotary evaporator (Eyela, USA) at 40°C until syrupy consistency was obtained. The concentrated extract was further dried at 35°C for 5 days inside an incubator, weighed, and kept in amber-colored container under 0°C until use.

C. Phytochemical Screening

Phytochemical screening was performed based on the procedure discussed [17]. Test for the presence of alkaloids, terpenes, glycosides, phenolic compounds and tannins were performed on the crude ethanolic leaf extract of *F. odorata*.

1. Dragendorff's and Meyer's Tests for Alkaloids

A small amount of the crude extract was acidified with 1% hydrochloric, boiled in a water bath and was filtered. The filtrate was divided into two portions. In a test tube, 1mL of the plant extract was added with freshly prepared Dragendorff reagent. Then, it was observed for the formation of orange precipitate. The other portion was treated with freshly prepared Mayer reagent and was observed for the formation of white precipitate.

2. Salkowski's Test for Terpenes

In a test tube, 2mL of the crude extract was mixed with 2mL of chloroform. Then, 2mL concentrated sulfuric acid was carefully added to the mixture. The test tube was observed for any red coloration on the chloroform layer.

3. Keller-Killani Test for Glycosides (2-deoxysugars)

Two milliliters of the crude extract was added with glacial acetic acid and a drop of 5% FeCl₃. Then, concentrated sulfuric acid was added carefully to the mixture. The test tube

was observed for reddish brown color at the junction and bluish green coloration of the upper layer.

4. Ferric Chloride Test for Tannins and Phenolic Compounds

Filtered crude extract was added with two drops of 5% FeCl₃ and was observed for deep blue black coloration.

D. Estimation of Total Phenolic and Total Flavonoid Content

1. Fast Blue BB Assay

The total phenolic content (TPC) of the crude extract was determined using Fast Blue BB assay as discussed [18]. A gallic acid standard curve was prepared together with the crude ethanolic leaf extract of *F. odorata*. In a 96-well microplate, 8.335μL aliquot of 0.1% Fast Blue BB reagent was added to the gallic acid standard and sample wells. Then, 8.335μL of 5% NaOH was added. The solution was incubated for 90 minutes at 25°C. After which, the plate was read at 420nm using Corona SH-1000 Microplate Reader (Hitachi, Japan).

2. Aluminum Chloride Assay

Aluminum chloride assay was performed as described [13] for the determination of total flavonoid content of the crude ethanolic leaf extract of *F. odorata*. Standard quercetin was used for the construction of the standard curve. In a tube, 60μL of the extract was added with 180μL distilled water and 18 μL 5% sodium nitrate. The mixture was then incubated for 5 minutes at 25°C. Then, 18μL 10 % (w/v) AlCl₃, 20μL 1mM NaOH and 204μL distilled water were added to the initial solution. The absorbance was read at 510 nm using Corona SH-1000 Microplate Reader (Hitachi, Japan).

E. Thin Layer Chromatography

Thin layer chromatography was performed to evaluate the components present in the crude ethanolic leaf extract of *F. odorata* based on the discussed procedure [13].

Small spots of the crude ethanolic leaf extract of *F. odorata* were applied to an activated TLC plate with aluminum backing (8cm x 5cm) together with the standard quercetin and gallic acid. The plate was allowed to develop in a glass chamber using toluene: ethyl acetate: methanol (6:3:1) as the solvent system. After the chromatogram development, the plate was air-dried and viewed under UV (254nm and 360nm). Lastly, the plate was further visualized using 0.05% methanolic DPPH solution.

F. Evaluation of Antioxidant Activity

1. DPPH Assay

Scavenging activity of the crude ethanolic leaf extract was evaluated using DPPH scavenging assay [13] and [16]. The assay employed the use of a stable free radical called 2,2-diphenyl-1-picrylhydrazyl and absorbs light at 517nm. Different concentrations of the ethanolic leaf extracts were prepared. In a 96-well microplate, 10μL of the crude extract

was added with 140µL of DPPH solution and the plate was incubated for 30 min at 25°C. The absorbance was read using Corona Microplate Reader SH-1000 (Hitachi, Japan).

2. FRAP Assay

Reducing power of the crude ethanolic leaf extract was determined using ferric reducing antioxidant (FRAP) assay [13]. This is based on the reduction of Fe (III) to Fe (II) through an electron transfer process in the presence of an antioxidant. High reduction capacity of the plant extract would signify that it has an antioxidant activity. Different concentrations of the extract were prepared. The reaction mixture will constitute the extracts, 1.0M hydrochloric acid (HCl), 1% sodium dodecyl sulphate (SDS), and 1% potassium ferric cyanide ($[K_3Fe_3(CN)_6]$). The tubes were incubated for 20min at 50°C. After incubation 0.1% ferric chloride ($FeCl_3$) was added to the solution and the absorbance was read at 750nm using Corona Microplate Reader SH-1000 (Hitachi, Japan).

G. Specific Free Radical Tests

1. Hydrogen Peroxide Assay

In a 96-well micropate, different concentrations of the crude ethanolic leaf extract (80µL) were loaded and 120µL of 40mmoles/L hydrogen peroxide was added on each well [19]. The plate was incubated for 10min at 25°C before the absorbance was read at 230nm using Corona Microplate Reader SH-1000 (Hitachi, Japan). The inhibitory activity of the crude extract was compared to that of the standard ascorbic acid.

2. Hydroxyl Radical Scavenging Assay

Scavenging of hydroxyl radicals was performed based on the procedure discussed [19]. In a small tube, 0.1mmole/L EDTA (20µL), 0.1mmole/L $FeCl_3$ (2µL), 2mmoles/L H_2O_2 (20µL), 3mmoles/L deoxyribose (72µL), phosphate buffer pH 7.4 (66µL), 0.1mmole/L ascorbic acid (20µL) and 200µL of the crude ethanolic leaf extract at different concentrations were mixed. The tubes were incubated for 30 min at 37°C. Then, 100µL of trichloroacetic acid (TCA) (5%w/v) and 100µL thiobarbituric acid (TBA) (1%w/v) were added to each tube. The tubes were then placed in a water bath for 30min. Lastly, the mixtures were transferred in a 96-well microplate in triplicates and the absorbance was read at 532nm using Corona Microplate Reader Sh-1000 (Hitachi, Japan).

3. Superoxide Anion Assay Assay

Different concentrations of the crude ethanolic leaf extract of *F. odorata* were mixed 73µmole/L NADH (50µL), 156µmole/L nitrobluetetrazolium (50µL) and 60µmole/L phenazine methosulfate (50µL) in a 96-well microplate. The plate was incubated for 5 min at 25°C before the absorbance was read at 560nm using Corona Microplate Reader SH-1000 (Hitachi, Japan) [13].

4. Nitric Oxide Assay

Following the procedure discussed [13], different concentrations of the crude ethanolic leaf extract of *F. odorata* was mixed with 10 mmole/L sodium nitroprusside and phosphate buffer saline (pH 7.4) in small tubes. The mixtures were incubated for 150 min at 25°C after which, 0.33% (w/v) sulfanilamide in 20% (v/v) acetic acid was added to the reaction mixture. The resulting mixtures were allowed to stand for 5min. Then, 0.1% (w/v) naphthylethylenediamine dihydrochloride was added and again incubated for 30 minutes at 25°C. The absorbance was read at 540nm using Corona Microplate Reader SH-1000 (Hitachi, Japan).

H. Statistical Analysis

The results was expressed as mean \pm SD. One-way analysis of variance (ANOVA) was used to determine if there are significant differences between the concentrations of the crude ethanolic leaf extract of *F. odorata* in scavenging free radicals and $p < 0.05$ was considered significant.

III. RESULTS AND DISCUSSIONS

A. Phytochemical Tests

The phytochemical test of the crude ethanolic leaf extract of *F. odorata* showed that the extract contains terpenes, glycosides and polyphenols. Terpenes and polyphenols are among the most studied and identified phytochemicals from plants. They provided several pharmacological and biological functions that are mostly utilized in health care development. Glycosidic forms of these two compounds are mostly common in plants. Terpenoid glycosides, such as cardiac glycosides, are often used in the treatment of various heart conditions due to its ability to regulate the Na^+/K^+ ATPase. On the other hand, polyphenolic glycosides are the ones responsible for the antioxidant activity of the plants. In addition to that, plant glycosides can also be a source of antibiotics and vitamins [20].

On the contrary, the crude extract was negative to alkaloids and flavonoids. As indicated in the study of Tsai et al. [21], the dichloromethane extract of *F. odorata* contained mainly of sterols and terpenoids that include I-sitosteryl-3- β -glucopyranoside-6'-O-palmitate, squalene, lutein, α -amyrin acetate, lupeol acetate and β -carotene.

B. Total Phenolic and Flavonoid Content

Fast Blue BB assay was used to estimate the total phenolic content (TPC) of the crude ethanolic leaf extract of *F. odorata*. Using a gallic acid standard curve, the TPC of 0.16mg/mL crude extract had 0.148µg/mL gallic acid equivalence (GAE). The aluminum chloride assay for total flavonoid content (TFC) estimation showed that the same concentration of the crude extract had a very low quercetin equivalence (QE) that is equal to 1.096×10^{-5} µg/mL.

The results suggest that the crude ethanolic leaf extract of *F. odorata* contains low amount of phenolic compounds and flavonoid as indicated by the phytochemical screening.

C. Thin Layer Chromatography

The TLC chromatogram of the crude ethanolic leaf extract of *F. odorata* showed 11 spots under visible light. However, none of these spots was able to correspond to the Rf values of gallic acid and quercetin standards that were used in the experiment. Moreover, the chromatogram was also viewed under ultraviolet light, both at 254nm and 360nm. The TLC chromatogram of *F. odorata* sprayed with 0.05% DPPH solution did not show any antioxidative components in contrast to quercetin and gallic acid standards. Positive result for the test would have been the change in color of the DPPH solution to yellow due to its reduction. The observed result may indicate that the separated components of *F. odorata* do not have a proton-donating ability as an antioxidant.

TABLE I
RF VALUES OF THE SPOTS OF THE CRUDE ETHANOLIC LEAF EXTRACT OF *F. ODORATA* OBTAINED FROM THIN LAYER CHROMATOGRAPHY

	Visible Light	254nm	364nm
S1	0.180 (light green)	0.180 (light green)	0.180 (pink)
S2	0.281 (light green)	0.281 (light green)	0.281 (brown)
S3	0.297 (green)	0.297 (green)	0.297 (green)
S4	0.344 (light yellow)	0.344(light yellow)	-
S5	0.414 (light yellow)	0.414(light yellow)	0.414 (brwn)
S6	0.453 (yellow)	0.453 (yellow)	0.453 (brown)
S7	0.828 (green)	0.828 (green)	0.828 (brown)
S8	0.844 (blue green)	0.844 (blue green)	0.844 (brown)
S9	0.891 (light brown)	0.891 (light brown)	0.891 (pink)
S10	0.953 (light brown)	0.953 (light brown)	0.953 (brown)
S11	0.989 (yellow)	0.989 (yellow)	0.989 (brown)
Quercetin	0.5	0.5	0.5
Gallic Acid	0.27	0.27	0.27

The TLC chromatogram was viewed under UV light, both at 254nm and 364nm. Quercetin and gallic acid were used as the standards

D. Evaluation of Antioxidant Activity

1. DPPH Assay

Proton-donating ability of the extract was evaluated through DPPH assay. It was shown in Fig. 1 that as the concentration of crude ethanolic leaf extract of *F. odorata* increase, there is an observed increase in the stimulation of DPPH radicals.

The compound 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable nitrogen-centered free radical that is commonly used to evaluate the antioxidant capacity of different substances [22]. In the presence of an antioxidant or a proton donor, DPPH is reduced to DPPH-H that shows a change in color from purple to yellow [23]. As indicted in the results, the crude ethanolic leaf extract of *F. odorata* does not have the ability to donate a proton that would reduce the DPPH radicals. This activity is in contrast to the reported activity of the crude ethanolic leaf extract of *F. pseudopalma*, which was a good scavenger of DPPH radicals [13]. Furthermore, the methanol: acetone extract of *F. bengalensis* aerial roots and *F. racemosa* stem bark were shown to exhibit a concentration-dependent inhibition of DPPH radical [14].

2. Ferric Reducing Antioxidant Power Assay

The reduction of potassium ferricyanide was used to measure the electron-donating ability of the crude ethanolic leaf extract of *F. odorata*. As observed in Fig. 2, there is a linear increase in the reducing power of the plant extract.

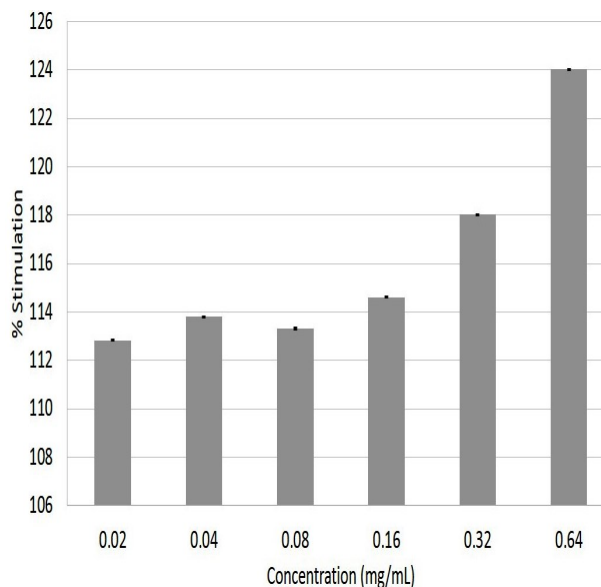


Fig. 1 Concentration-dependent stimulation of DPPH radicals by the crude ethanolic leaf extract of *F. odorata* (n=3; p<0.05)

The ferric reducing activity of the crude ethanolic leaf extract of *F. odorata* was measured via the direct reduction of ferricyanide ions to ferrocyanide, which involves an electron transfer from an antioxidant [24]. Based on the results, though the extract's reducing power was observed to be concentration-dependent, this activity is considered to be low as compared to that of the standard ascorbic acid and even from its sister plants, *F. pseudopalma* [13] and [16] *F. callosa*, *F. vasculosa*, *F. auriculata* and *F. virens* var. *verins* [25]. On the contrary, related study showed that *Ficus racemosa* and *Ficus oligodon* [25] were found to have quite similar reducing activity as that of *F. odorata*.

The assessment of both the proton- and electron-donating ability of the plant extracts is good measure of the plant's antioxidant capacity. However, the results showed that *F. odorata* has a very low antioxidant activity.

In this regard, other specific tests were performed in order to determine possible action to some of the biologically important species and relate these actions to particular medical applications.

F. Stimulation of Superoxide, Hydrogen Peroxide and Hydroxyl Radicals

Superoxide anions ($\bullet\text{O}_2^-$) are one of the ROS that are normally produced inside the body. The formation of these molecules by phagocytic immune cells is catalyzed by a membrane-bound NADPH oxidase and is utilized for the killing of invading microorganisms and other pathogens [26]

and [27]. Thus, controlled production of $\bullet\text{O}_2^-$ is essential in maintaining the healthy physiological environment.

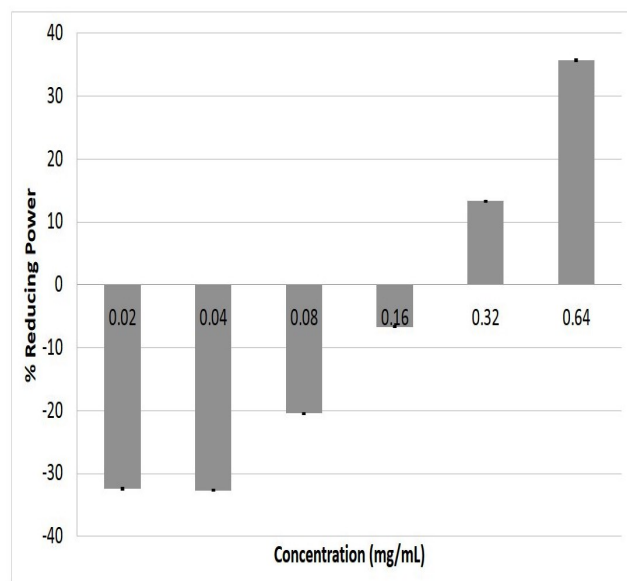


Fig. 2 Ferric reducing ability of the crude ethanolic leaf extract of *F. odorata* (n=3; p<0.05)

Hydrogen peroxide (H_2O_2) is biologically produced in cells and is used for host defense mechanism and some oxidative biosynthetic reactions [28]. Moreover, H_2O_2 it has also been regarded as a signaling molecule. At low concentrations, H_2O_2 was shown to stimulate the proliferation of mammalian cells as well as induction and upregulation of specific genes and proteins [29]-[33].

In the study, the crude ethanolic leaf extract of *F. odorata* was shown to stimulate both the production of $\bullet\text{O}_2^-$ and H_2O_2 in a concentration-dependent manner (Figs. 3 and 4). This stimulatory activity of *F. odorata* is also comparable to the observed activity of *F. pseudopalma* as discussed [13]. Stimulation of hydrogen peroxide was also observed in some plants. A study had shown that the stimulation of H_2O_2 production by green and oolong tea extracts can be useful in the inhibition of leukemic human leukocytes *in vitro* [34].

The result may suggest that the crude ethanolic leaf extract *F. odorata* has a potential in aiding phagocytes in maintaining the body's immune defense against pathogenic invaders as well as promote other biologically important functions.

In one related study, $\bullet\text{O}_2^-$ and H_2O_2 had been linked to the induction of tumor cell apoptosis [35]. Based on the study, H_2O_2 was involved on the UV irradiation-induced apoptosis in HL-60 cells that increased the intracellular peroxide levels as detected by fluorometric method. On the other hand, $\bullet\text{O}_2^-$ may have been an intermediate in the production of H_2O_2 and hydroxyl radicals ($\bullet\text{OH}$) that explained its indirect involvement in the death of HL-60 cells.

As discussed by Halliwell [36], the toxicity observed in O_2^- has been associated to the production of reactive hydroxyl

radical ($\bullet\text{OH}$) when it reacts with H_2O_2 and in the presence of Fe^{2+} ions. Known as an extremely reactive free radical in the body, $\bullet\text{OH}$ can promote severe damage in living cells [14]. However, their reactive nature promotes the degradation of foreign substances and maintains cellular homeostasis within the cells.

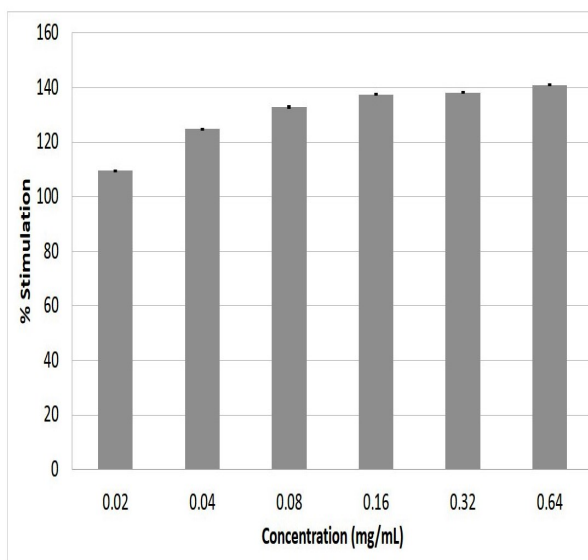


Fig. 3 Concentration-dependent stimulation of superoxide anions by the crude ethanolic leaf extract of *F. odorata* (n=3; p<0.05)

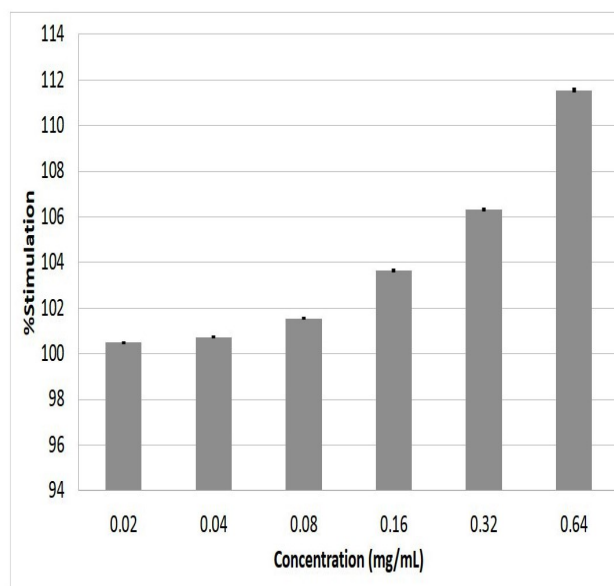


Fig. 4 Stimulation of hydrogen peroxide by the crude ethanolic leaf extract of *F. odorata* (n=3; p<0.05)

Fig. 5 shows that the crude ethanolic leaf extract of *F. odorata* has a concentration-dependent stimulation of hydroxyl radicals. This activity was observed to be similar to that of *F. pseudopalma* [13].

G. Stimulation of Nitric Oxide

The crude ethanolic leaf extract of *F. odorata* was able to stimulate the production of nitric oxide in a concentration-dependent manner as shown in Fig. 6.

Nitric oxide (NO•) is a gaseous lipophilic free radical [37] that is involved in numerous physiological processes [38]. It was identified as a relevant factor in endothelial dysfunction [39]. NO deficiency had been associated to several coronary diseases such as hypertension, coronary heart disease and heart failure [40].

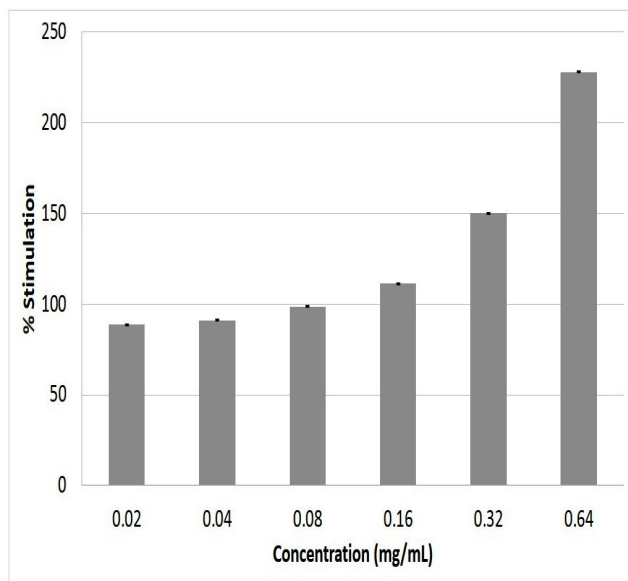


Fig. 5 Concentration-dependent stimulation of hydroxyl free radicals by the crude ethanolic leaf extract of *F. odorata* (n=3; p<0.05)

NO has a cytoprotective as well as cytotoxic roles. Its cytotoxic activity is related to the production of peroxynitrite ions when it reacts with O₂ ions. This is often used as a defense mechanism in killing pathogens by promoting DNA or protein damage [41]. In addition to that, NO is also involved in bone development and remodeling. As discussed in the review of Grant and El-Fakahany [38], it was found that NO is important both in the regulation of osteoblast and osteoclast activities.

Non-adrenergic non-cholinergic stimulation of penile erection also involves that vasodilatory function of NO [42]. Regulatory levels of NO are also involved in obstetrics, rheumatoid arthritis and postmenopausal osteoporosis [38].

In addition to the biological activities that *F. odorata* may have in relation to the function of each of the molecules discussed, its pro-oxidant activity may also be helpful in the treatment of cancer. In an article [43], they deliberated the possible activity of the secondary metabolites produced by some natural products that can be linked to their anticancer properties. Oxidative stress is induced by pro-oxidant agents by producing ROS or inhibiting the antioxidant systems [44]. Since cancer cells are usually anaerobic in nature,

augmentation of ROS levels produce much oxidative stress that most of the cancer cells may not tolerate anymore. Over production of ROS can lead to oxidative damage that may lead to protein and lipid structure modifications as well as chromosomal aberrations [45]-[47]. Thus, the prooxidant nature of *F. odorata* can be used for anticancer applications.

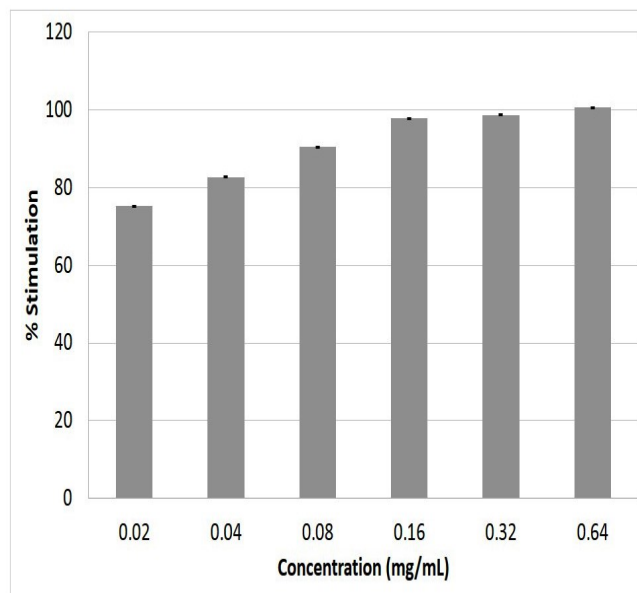


Fig. 6 Concentration-dependent stimulation of nitric oxide by the crude ethanolic leaf extract of *F. odorata* (n=3; p<0.05)

IV. CONCLUSION

In relation to its stimulatory activity towards hydrogen peroxide, hydroxyl radicals, superoxide anions and nitric oxide, *F. odorata* can be developed as an agent with several important biological and pharmacological applications that can be useful in the treatment of cardiovascular disease, erectile dysfunction and impotence, cancer and aging. Also, it can aid in the maintenance of the immune defense mechanism of the body thus, conserve the homeostatic and healthy cellular environment. Lastly, its prooxidant activity can also become useful for the treatment of cancer.

In this regard, *F. odorata* can be tapped as a functional food and for nutraceutical and drug development with interesting biological and pharmacological functions by our local food and drug companies.

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