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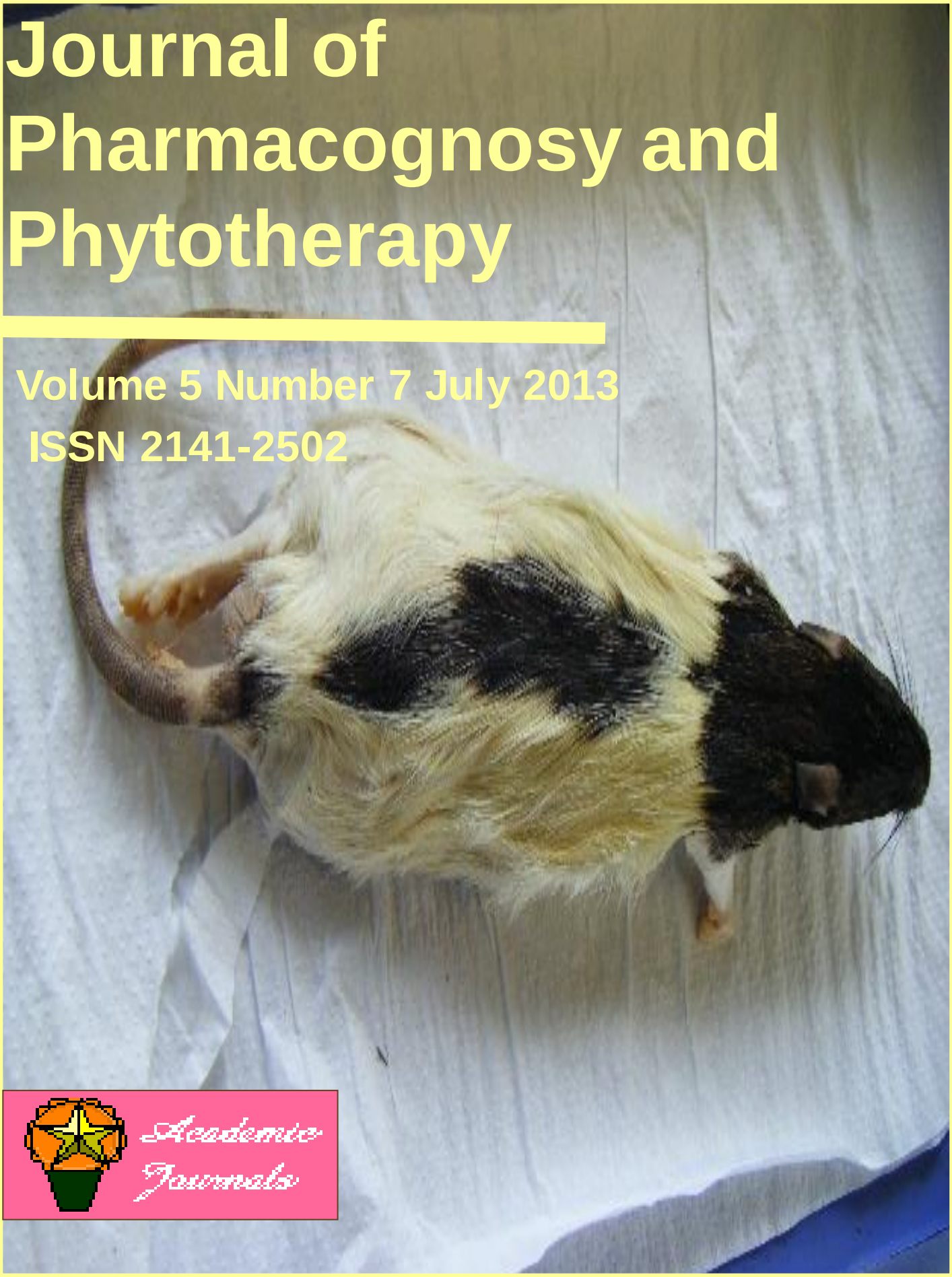

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Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds), Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International. pp 181-190.

Jake OO (2002). Pharmaceutical Interactions between *Striga hermonthica* (Del.) Benth. and fluorescent rhizosphere bacteria Of *Zea mays*, L. and *Sorghum bicolor* L. Moench for Striga suicidal germination In *Vigna unguiculata*. PhD dissertation, Tehran University, Iran.

Furmaga EM (1993). Pharmacist management of a hyperlipidemia clinic. Am. J. Hosp. Pharm. 50: 91-95

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Table of Contents: Volume 5 Number 7 July 2013

# ARTICLES

## Research Articles

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Journal of Pharmacognosy and Phytotherapy

*Full Length Research Paper*

# Evaluation of retinoblastoma (Rb) and protein-53 (p53) gene expression levels in breast cancer cell lines (MCF-7) induced with some selected cytotoxic plants

**Samuel Titilola Aderonke[1]*, James Ayorinde Babatunde[1], Oshodi Temitope Adesola[2], Odii Uchennaya Okereke[1], Chidume Innocent[1], Makinde Oluwaseun Elisha[1], Oguntoye Lanre Abolaji[1] and Magbagbeola Olubunmi Abiola[1]**

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**Many Nigerian plants have been hypothesized to have anticancer potentials. However, not many of them have been subjected to acceptable scientific evaluation for their potential anticancer effects. In this study, six of such plants were selected to evaluate the effects of their crude, hexane, chloroform, ethylacetate, detanninfied and tannin fractions for brine shrimp lethality assay and the most cytotoxic fractions of each plant were further tested on gene expressions of TP53 and retinoblastoma (Rb) genes in human breast cancer cell line (MCF-7). *Gladiolus psittacinus* (Gps), *Icacina trichantha* (Itr), *Spilanthes filicaulis* (Sfi), *Curculigo pilosa* (Cpi), *Anthocleista djalonensis* (Adj), and *Tapinanthus bangwensis* (Tba) medicinal plants were selected for this study. Crude extracts of 80% aqueous ethanol macerated plant materials were fractionated into hexane, chloroform and ethylacetate fractions. The resultant aqueous fractions were detanninfied to produce aqueous detanninfied fractions and tannin fractions. The 36 panel of plant fractions produced from all the plants were used for the study. From our findings, hexane fraction of *S. filicaulis* (Sfi-HF) showed the highest cytotoxic effect ($LC_{50}$ 21.30 µg/ml) on brine shrimps showing a low signal of p53 gene expression but a high intensity of Rb gene expression in MCF-7 cell lines. Moreso, crude extract of *G. psittacinus* (Gps-CE) showed a significant ($P<0.05$) increase in TP53 gene expression in comparison with the control group and also a high intensity of Rb gene expression. Our results demonstrates the modulatory potentials of Sfi-HF and Gps-CE on TP53 and Rb gene expressions in MCF-7 breast cancer cell lines suggesting a possible mode of action of Sfi-HF and Gps-CE amongst a panel of 36 extract fractions.**

**Key words:** TP53 gene, retinoblastoma (Rb) gene, brine shrimps, cytotoxicity, gene expression, medicinal plants.

## INTRODUCTION

Among the various cancer types, breast cancer is the most common cause of cancer deaths. Statistics indicates that about 1.3 million women are diagnosed with breast cancer annually worldwide and about 465,000 die from the disease (Elangovan et al., 2008). The elucidation and molecular mechanisms underlying neoplastic transformation and progression have resulted in the understanding that breast cancer can be regarded as a genetic disease, which evolved from the accumulation of a series of acquired genetic lesions (Stoff-Khalili et al.,

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**Table 1.** List of medicinal plants used in this study.

| S/N | Scientific name | Common/Local name | Plants' part used |
|---|---|---|---|
| 1 | *Tapinanthes bangwensis* | Mistletoe/Afomo | Leaves |
| 2 | *Spilanthes filicaulis* | Brasil cress/Awere pepe | Leaves |
| 3 | *Gladiolus pscittanus* | Dragon head lily/Baaka | Bulbs |
| 4 | *Icacina trichantha* | False Yam/Gbegbe | Leaves |
| 5 | *Anthocleista djalonensis* | Cabbage tree/Ewesapo | Leaves |
| 6 | *Curculigo pilosa* | African crocus/Epakun | Rhizome |

2006). These genetic lesions lead to inactivation of tumor suppressor genes and/or activation of oncogenes (Stoff-Khalili et al., 2006).

An example of tumor suppressor activation in breast cancer is that of retinoblastoma (Rb1) (Lee et al., 1988), which is seen amplified and under-expressed in 10 to 15% of breast tumors, and in some series, has been associated with a worse prognosis or more aggressive clinical features (Osborne et al., 2004). TP53 is another tumour suppressor gene that functions to eliminate and inhibit proliferation of abnormal cells by inducing apoptosis and/or activating deoxyribonucleic acid (DNA) repair mechanisms, thereby preventing tumour/cancer development. TP53 gene has been shown to be the most commonly mutated gene in many common human cancers, with mutations estimated to occur in 50% of all cancers (Sigal and Rotter, 2000). Under normal cellular conditions, the p53 signaling pathway operates in ‘‘standby’’ mode. However, in the presence of cellular stresses such as DNA damage and expression of activated oncogenes, the signalling pathway is activated (Vogelstein et al., 2001). High levels of p53 protein are a common phenomenon in many human neoplasia (Vojtesek and Lane, 1993). Several studies have showed a close correlation between mutation of the TP53 gene and accumulation of high levels of p53 tumours (Troester et al., 2006; Vojtesek and Lane, 1993; Bennet et al., 1991; Davidoff et al., 1991). In early studies, expression of mutant p53 was demonstrated in breast cancer cell lines (Bartek et al., 1990).

However, studies have shown that TP53 gene is frequently inactivated by mutation or other mechanisms in human breast cancer, but only occurs in 30% of breast cancers (Alkahaf and El-Mowafy, 2003; Borresen-Dale, 2003; Olivier and Hainut, 2001). Gene expression *in vitro* studies have demonstrated that cell line models of luminal breast cancers show notable changes in $P_{53}$ genes such as p21, but the same magnitude of p53 regulated responses was not observed for cell line models of basal like breast cancer (Troester et al., 2006). Therefore, a luminal epithelial breast cancer cell line, MCF-7 was used in this study which is known to habour wild-type (normal) TP53 gene.

Bioactives from natural products are emerging strategies in suppressing tumours by inducing apoptosis (Alshatwi, 2011). Vinca alkaloids and taxoids are one of the earliest and major natural product derived drugs in mainstream cancer treatment (Warber et al., 2006). In addition, more phytochemical such as camptothecin and podophyllotoxin were discovered and many of these natural compounds were structurally modified to yield stronger anti-cancer analogues with less adverse effects (Azizi et al., 2009). In essence, it is important to investigate the mechanism of action of proposed antitumour phytochemicals in which understanding the modulation of gene expressions of oncogenes and tumour suppressor genes are key to tumour development. Based on published literatures and folklore in Nigeria, six plants (*Tapinanthes bangwensis* (Tba), *Spilanthes ficaulis* (Sfi), *Anthocleista djalonensis* (Adj), *Curculigo pilosa* (Cpi), *Icacina trichantha* (Itr) and *Gladiolus psittacinus* (Gps)) hypothesized to have antitumour properties were investigated for their cytotoxic activity on brine shrimps and modulatory property on p53 and Rb genes.

## MATERIALS AND METHODS

Plant materials used as listed in Table 1 were bought from Awolowo Market, a reputable herbal Market in Mushin Local Government Area of Lagos State, Nigeria. They were identified at the Herbarium Unit of Botany Department, University of Lagos, Lagos State, Nigeria, and voucher specimens were deposited.

### Preparation of plants' crude extracts

Plant materials were cut to increase surface area and were dried at 40°C for 3 days. After drying, 200 g of plants were pulverized and weighed in 500 ml 70% aqueous-ethanol to soak the pulverized plants. After 14 days of marceration, the residual plant materials (marc) were separated from the solvent by filtration over a muslin bag. The filtrates were further filtered using low ash filter papers. The crude extracts were concentrated using a hot-air oven at temperature of 45°C (Siedel, 2006).

### Organic solvents and column partitioning of crude extracts

The aqueous-ethanol extracts of the plant samples were individually fractionated by organic solvent extraction (n-hexane, chloroform and ethylacetate). Five grams each of concentrated crude extracts were dissolved in 20 ml deionized water and 70 ml of the organic solvent (n-hexane) was added and the immiscible

mixture was transferred into a separatory funnel. The resultant suspension was mixed vigorously and subsequently allowed to phase separate in order to separate the aqueous and organic fractions. After each separation, other organic solvents were sequentially added to the aqueous fractions for further partitioning into different organic solvents (Otsuka, 2006).

Polyphenolic compounds from the aqueous fractions were separated by passing 20 ml of 50 mg/ml aqueous fractions over 6 g polyamide column to trap the polyphenols. The column bound polyphenolic compounds were released by elution with 100 ml of absolute methanol (Collins, 1998).

#### Brine shrimps (*Artemia salina*) lethality assay

Brine shrimp cytotoxicity assay (Sowemimo et al., 2007) was used to screen the thirty six (36) fractions (crude extract, n-hexane, chloroform, ethylacetate, detannified, and tannin fractions for each of the six plants). One milliliter of each stock solution (50, 500, and 5000 µg/ml) was put into a test tube and made up to 5 ml with filtered sea water to give overall concentrations of 10, 100 and 1000 µg/ml. Ten brine shrimp larvae were then placed in each of the test tubes. After 24 h, the number of shrimps which survived were counted using a magnifying lens and recorded. All experimental assays were done in triplicates. The $LC_{50}$ was calculated using the Probit method (Wardlaw, 1985).

#### Cell culture and treatment

MCF-7 cell lines were grown in 7 Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (FCS). Cells were incubated in a humidified 37°C, 95% $O_2$ + 5% $CO_2$ incubator and the medium was continuously changed every 2 to 3 days interval to feed the culture. At 70% confluence, $0.1 \times 10^5$ cells were seeded into 24 well plates in DMEM containing 2% FCS (maintenance medium). Seven fractions with the lowest brine shrimp lethality $LC_{50}$ values out of the thirty six (36) fractions (with at least one fraction representing each plant) were selected. A concentration that is five-fold lower than their $LC_{50}$ values were prepared in maintenance medium (inoculating medium). One milliliter of the inoculating medium was added to each well in replacement of the maintenance medium. The first three wells were used as the control (DMEM only) while the other wells contained inoculating medium of the selected fractions in triplicates.

#### RNA extraction and cDNA synthesis

At the end of 24 h incubation, cells were harvested and total RNA was extracted from each sample. The RNA was extracted according to Qiagen RNeasy kit (USA). Extracted RNA samples were quantified using Nanodrop spectrophotometer. 60 ng of normalized RNA samples were converted into cDNA in a reaction volume of 25 µl comprising of 500 ng oligodT, 1X Script buffer, 0.1 mM DTT, 1 U/µl Rnase inhibitor, 0.4 mM dNTP, 4.0 U/ul reverse transcriptase enzyme. PCR condition was done as described in the Jena Bioscience SCRIPT® Reverse transcriptase kit (Germany).

#### *Gene quantitation*

Polymerase chain reaction **(**PCR) was performed in a 25 µl reaction volume containing 5 µg of cDNA, 0.2 mM dNTP mix, 1X complete buffer (Jena Biosciences), 0.04 U/µl high yield taq polymerase (Jena Bioscience), 0.5X Sybr green I (Invitrogen, Germany) and 0.5 µM of each target primer pair. Thermal cycling was done at 94°C for 2 min; 94°C for 30 s; 56°C for 30 s; 72°C for 30 s; and 72°C for 2 min; step 2 to 4 was repeated 35 times. Primers pairs used in this study were *β-actin* fwd/rvs (5'-GGC ATG GGT CAG AAG GAT TC-3'/5'- ACA TGA TCT GGG TCA TCT TCT C-3'); *TP53* fwd/rvs (5'-GCG CAC AGA GGA AGA GAA TC-3'/5'-CAA GGC CTC ATT CAG CTC TC-3'); *Rb* fwd/rvs (AGGACCGAGAAGGACCAACT/AAGGCTGAGGTTGCTTGTGT). *β-actin* gene was used as the internal control (endogenous gene). PCR amplicons were run on 1.8% agarose gel electrophoresis in Tris-Acetate EDTA buffer at 120 V for 25 min. Gel images were captured and analyzed using Gelanalyzer.

#### Data analysis

The percentage lethality of the extracts and fractions on brine shrimps was calculated from the mean of larvae that survived in the treated tubes and controls. The numbers of dead nauplii was divided by initial number of nauplii (10) and multiply by 100. Finney's probit analysis was used to determine the $LC_{50}$ of each extract at 95% confidence interval.

Relative densitometric intensity of the PCR amplicon bands was done using Gelanalyzer software. The intensity values were computed into Graphpad Prism 5© software to calculate the significant differences between the control and test groups at 95% confidence interval.

## RESULTS AND DISCUSSION

The brine shrimp lethality test is a simple bioassay used for the primary screening of the crude extract of plant as well as isolated compounds to assess toxicity towards brine shrimp, which could also provide an indication of possible cytotoxic properties of the test materials (Meyer et al., 1982). A number of novel antitumor and pesticidal natural product have been isolated using this bioassay (McLaughlin et al., 1991). In this investigation, the extract and fractions of plants such as Tba, Sfi, Adj, Cpi, Itr and Gps were screened to assess their toxicity towards brine shrimps. After screening, the six most cytotoxic test agents with their respective $LC_{50}$ values out of the thirty-six are hexane fraction of Sfi (21.30), aqueous detanninfied fraction of Itr (66.08), aqueous detanninfied fraction of Adj (74.12), crude extract of Gps (82.68), hexane fraction of Tba (223.46), and crude extract of Cpi (274.20) as shown in Table 2. Bassey et al. (2012) reported that hexane fraction of Tba showed considerable cytotoxicity against brine shrimps.

Also, from the results, it was found that the crude fraction exhibited the highest cytotoxic activity for most of the plants, while the tannin fraction showed no significant cytotoxic activity for almost all the plants. The high cytotoxicity of the crude fraction might be as a result of a combination of bioactive compounds, both lipophilic and hydrophilic, present in the crude fraction, which probably enhanced its cytotoxic activity. On the other hand, the low cytotoxicity of the tannin fractions could be as a result of breakdown of tannins by the brine shrimps, which probably resulted in their low cytotoxicity. This is justified by the work of Makkar et al. (1995), who reported that invertebrates have the ability to secrete binding polymers

**Table 2.** Brineshrimp lethality assay of test fractions.

| S/N | Test fraction | $LC_{50}$ values on *Artemia salina* | | | | | |
|---|---|---|---|---|---|---|---|
| | | ***Icacina trichantha*** | ***Curculigo pilosa*** | ***Spilanthes ficaulis*** | ***Anthocleista djalonensis*** | ***Tapinanthes bangwensis*** | ***Gladiolus psittacinus*** |
| 1 | Crude extract (CE) | 185.68 | 274.20 | 30.87 | 148.78 | 993.78 | 82.68 |
| 2 | Hexane fraction (HF) | 1046.72 | 1640.65 | 21.30 | 154.74 | 223.46 | 4340.62 |
| 3 | Chloroform fraction (CF) | 319.47 | 964.57 | 444.30 | 426.15 | 904.78 | 475.46 |
| 4 | Ethylacetate fraction (EF) | 615.85 | 94037.10 | 51.57 | 103.65 | 864.98 | 114.52 |
| 5 | Aqueous detannified fraction (DF) | 66.08 | 479.75 | 646.54 | 74.12 | 1522.50 | 121.65 |
| 6 | Aqueous tannin fraction (TF) | 94037.10 | ND | 2046.28 | ND | 60519.18 | 140266.50 |

Lowest $LC_{50}$ values of each plant are shaded. ND= Not detected.

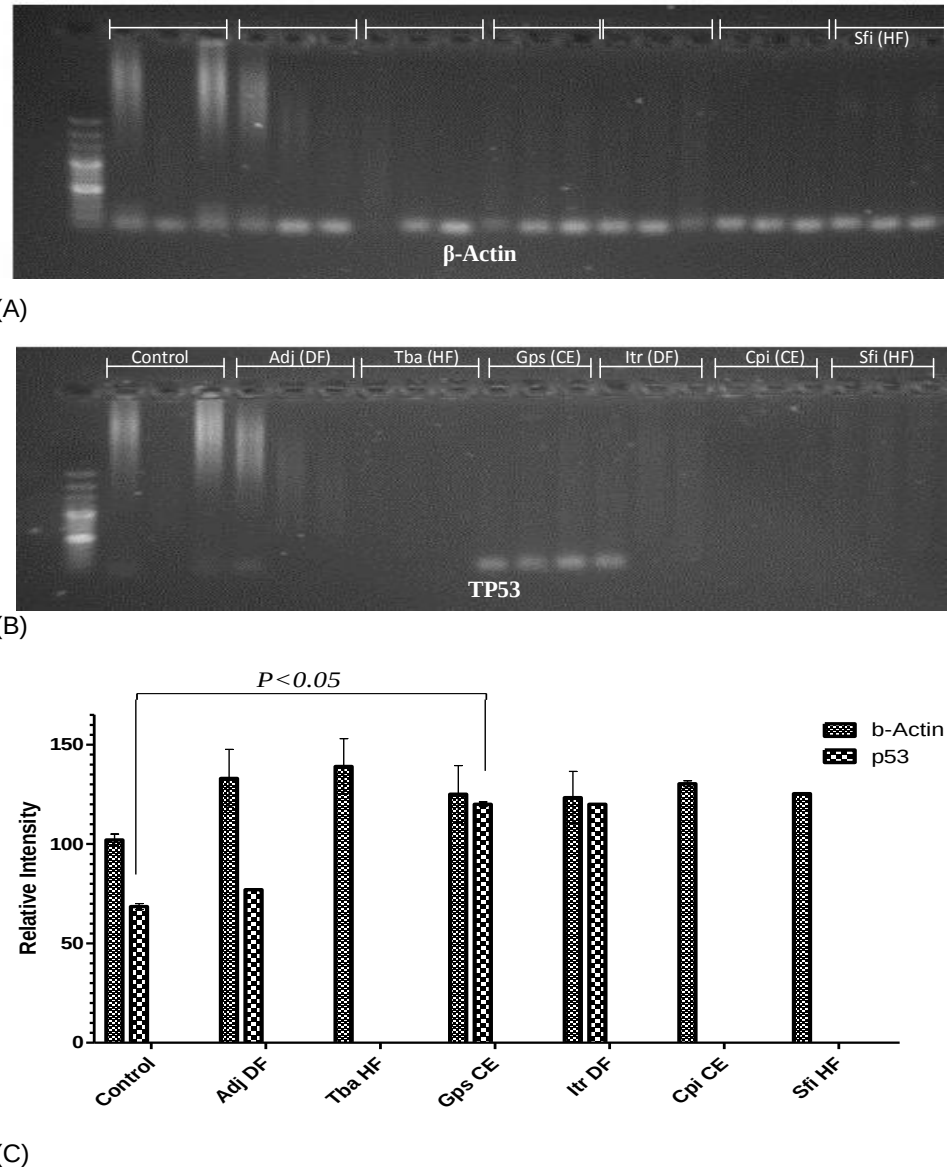
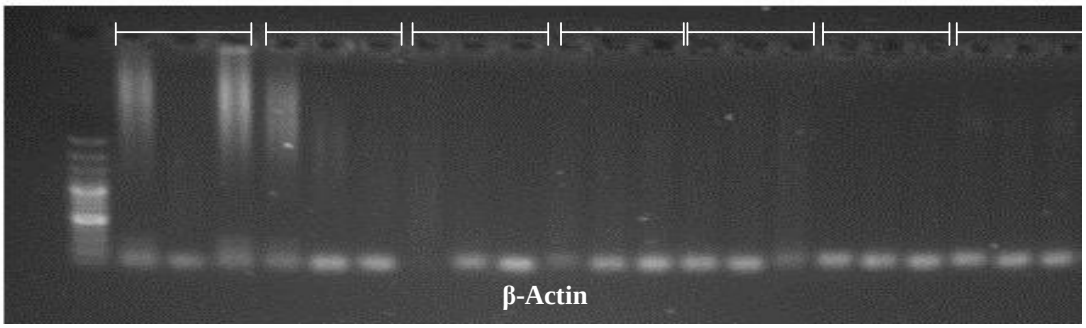
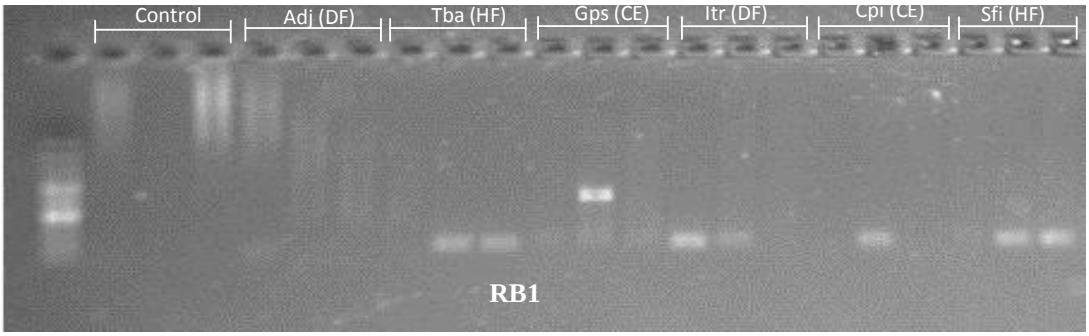



**Figure 1.** Densitometric analysis of RT-PCR amplicons for (A) β-Actin gene 1.8% agarose gel, (B) TP53 gene 1.8% agarose gel, and (C) error bar charts showing relative intensities of each band; testing was done in triplicates and values are Mean ± standard error (SE). P<0.05 is considered significant versus control group.




(A)

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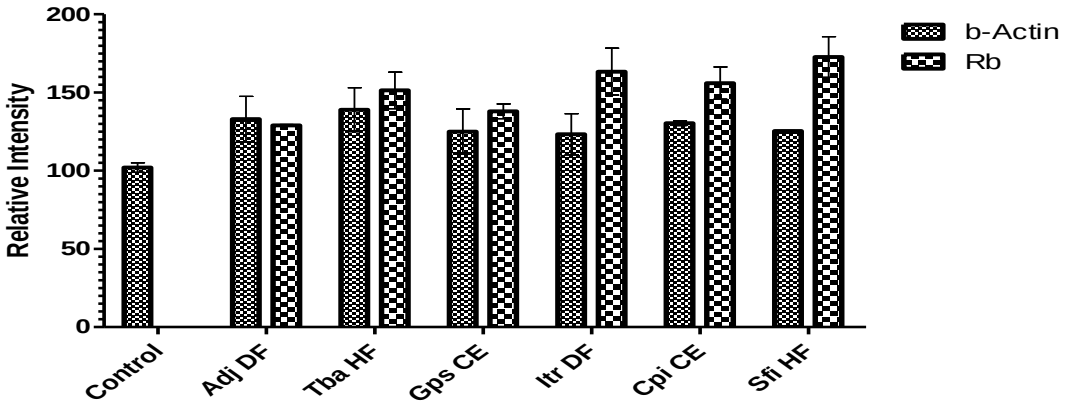


(C)

**Figure 2.** Densitometric analysis of RT-PCR amplicons for (A) β-Actin gene 1.8% agarose gel, (B) RB1 gene 1.8% agarose gel, (C) error bar charts showing relative intensities of each band; testing was done in triplicates and values are Mean ± standard error (SE). P<0.05 is considered significant versus control group.

and tannin-resistant enzymes as defense mechanisms against tannins which could lead to its biodegradation, thereby reducing its cytotoxic activity.

In the gene expression assay, the breast cancer cell line MCF-7 was treated with concentrations five-fold lower than the $LC_{50}$ values of each of the six most cytotoxic fractions selected for 48 h and the expression levels of TP53 and RB1 genes were evaluated. The retinoblastoma tumour suppressor gene (RB1) encodes a nuclear phosphoprotein that plays a central role in regulating the cell cycle in which its increase in expression levels can induce apoptosis (Weinberg et al., 1995; Reed, 2000). As shown in this study (Figure 2), all the cytotoxic fractions caused an upregulation of the RB1 gene with the hexane fraction of Sfi showing the highest followed by the aqueous detannified fraction of Itr. Retinoblastoma protein, pRb participates in a mechanism that arrests cell division in G1 when DNA damage is

detected. Unphosphorylated pRb binds the transcription factor E2F; while when bound to pRb, E2F cannot promote transcription of a group of genes necessary for DNA synthesis (DNA polymerase α, ribonucleotide reductase, thymidine kinase, proliferating cell nuclear antigen, and RAD51) (Nevins, 2001).

Several authors have investigated the effects of phytochemicals on TP53 gene expression as a measure of apoptosis induction in cell lines (Azizi et al., 2009; Yaacob et al., 2010; Wang and Sun, 2010; Alshatwi et al., 2011; Alshehri and Elsayed, 2012). It is well known that p53 acts biochemically as a transcription factor and biologically as a powerful tumor suppressor. Under normal, unstressed conditions, p53 protein remains undetectable due to its short half-life. The p53 instability is primarily controlled by its negative regulator Mdm2, which, as an E3 ubiquitin ligase, targets p53 for proteasome-mediated degradation (Wang and Sun, 2010). In this study, aqueous detanninfied Itr, Adj and crude extract of Gps caused upregulation of p53 gene as shown in Figure 1. Crude extract of Gps produced a significant ($P<0.05$) change in comparison with the control group. Samuel et al. (2011) reported the hepatoprotective ability of *Icacina trichantha* (Itr) sodium arsenite induced genotoxicity suggesting it as a possible anti-tumour agent. The observed increased expression in the treatment with *Itr*-DF, *Adj*-DF and *Gps*-CE suggests that the fractions contain bioactive compounds that restored the loss of function of p53 and Rb in the breast cancer cell line, MCF-7.

In conclusion, our results demonstrate the modulatory potentials of Sfi-HF on Rb and Gps-CE on both p53 and Rb gene expressions in MCF-7 breast cancer cell lines suggesting a possible mode of action of Sfi-HF and Gps-CE amongst a panel of 36 extract fractions.

## ABBREVIATIONS

**CE**, Crude extract; **HF**, hexane fraction; **CF**, chloroform fraction, **EF**, ethylacetate fraction; **DF**, aqueous detanninfied fraction; **TF**, tannin fraction.

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*Full Length Research Paper*

# Pharmacognostic and hypoglycemic studies of *Achyranthus aspera* L.

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**In the present study, the ethanolic extract of *Achyranthus aspera* L. Leaves (EEA) were investigated for their pharmacognostic characteristics and hypoglycemic effects. The pharmacognosy of the plant was carried out using microscopic and macroscopic tools. Macroscopic studies revealed that the leaves were cauline, ramel, opposite, exstipulate, simple, sub-sessile, ovate, entire, acute, unicostate, reticulate, rough, coriaceous and hairy. The stem was erect, herbaceous, quadrangular, branched, solid, green and pubescent, while various internal parts were observed using powder drug for microscopic study. The hypoglycemic effect of the plant was studied in healthy normoglycemic rabbits. The EEA was tested in three doses (100, 150 and 200 mg/kg), and a dose dependent hypoglycemic effect was observed. The most significant hypoglycemic effect was observed against higher dose (200 mg/kg) which remained for 5 h while the hypoglycemic effect remained significant for 3 h against the lower dose. It was concluded that EEA has hypoglycemic properties and it is recommended for the treatment of diabetes mellitus II.**

**Key words:** Pharmacognostic, hypoglycemic, *Achyranthus aspera* L.

## INTRODUCTION

*Achyranthus aspera* L. is a common perennial herb, growing as a wild plant, in natural and cultivated habitats. *A. aspera* L. has a branched tap root system. The stem is herbaceous, quadrangular, branched, solid, green and pubescent. Leaves are simple and exstipulate. A spike type of recemose inflorescence occurs in this specimen. Almost all parts (seed, root, stem and leaf) of *A. aspera* L. possess medicinal value (Lans, 2007) but leaves have greater importance in the traditional (unani) system of health and herbal medicine formulation. Alcoholic decoctions of *A. aspera* L. are mostly used as anti-inflammatory (Girach Aminuddin and Khan, 1992) and infertility inducing agents (Chakraborty et al., 2002; Shibeshi et al., 2006). Furthermore, the plant possesses lavicidal (Bagavan et al., 2008), antioxidant (Edwin et al., 2008), anticancer (Chakraborty et al., 2002), anti- diarrheal and antidysentric (Boily and Van Puyvelde
, 1986; Girach Aminuddin and Khan, 1992), antimicrobial (Boily and Van Puyvelde, 1986) properties. The hypoglycemic effect of methanolic and aqueous extract of the whole plant of *A. aspera* has been reported (Akhtar and Iqbal, 1991). The pharmacognostic profile and hypoglycemic effect of the leaves of this plant was studied in order to validate traditional uses.

## MATERIALS AND METHODS

### Plant

The study was conducted in the Department of Botany, University

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of Peshawar. Plants of *A. aspera* was collected from different localities in the district of Charsadda, KPK, Pakistan. Plants were identified by Dr. Muhammad Ibrar, Department of Botany, and specimen was deposited there in the herbarium under voucher number 7455/Bot. The whole plant was powdered for microscopic study.

#### Preparation of extract

The ethanolic extract of the leaves was prepared using our previous reported method (Barkatullah et al., 2011; Muhammad and Saeed, 2011; Muhammad et al., 2012b, c). Powder of leaves was macerated in ethanol for 10 to 15 days. The extract was filtered and then evaporated. The dry extract was stored in refrigerator for use.

#### Animals

Domesticated rabbits weighing 1 kg obtained from the district Charsadda District, Pakistan were used. The animals were kept at standard laboratory conditions. The animals were shifted from animal house to the laboratory one hour prior to start the experiment.

#### Pharmacognostic study

The pharmacognostic profile of the plant was reported using our previous reported method (Muhammad et al., 2012; Ismail et al., 2011). Vein islet number, vein termination number, palisade ratio, stomatal number and stomatal indices were calculated from 1 $mm^2$ pieces of leaf of *A. aspera* boiled in concentrated chloral hydrate solution under high power compound microscope.

#### Macroscopical features

The following macroscopic characters of plant were noted: size, shape, margins, apex, surface, color, odour, taste, nature, texture of leaves, stem and root were also studied (Wallis, 1985; Evans and Trease, 2002).

#### Powder drug Study

Powdered drug was studied using chloral hydrate and iodine solution under compound microscope (Compoud microscope). Organoleptic evaluation (color, odour, taste, fracture and touch) of the powdered drug was done using five senses (Pharmacopoeia, 1986).

#### Phytochemical Study

##### *Qualitative chemical identification tests*

Various phytochemical tests were performed for detection of various constituents preliminary, using well established procedures (Trease and Evans, 1989; Muhammad and Saeed, 2011).

##### *Hypoglycemic effects*

The healthy rabbits were divided into five groups of six. Group I was treated with normal saline (10 ml/kg) and acted as a control, Group II was treated with the standard hypoglycemic drug, glibenclimide, Group III, IV and V were treated with the ethanolic extract of Achyranthus aspera (EEA) at doses of 100, 150 and 200 mg/kg, respectively. Blood glucose level was noted at the start of the experiment and then after 1, 2, 3, 4 and 5 h, respectively. Glibenclimide was used as a standard hypoglycemic drug. Swab soaked in methylated spirit was used as a disinfectant during collection of blood. Blood was obtained by puncturing the veins in the pinna of each animal by using a sharp lancet . A swab soaked in methylated spirit was used to disinfect the lancet after each use.

## RESULTS

### Macroscopical features

Leaves of *A. aspera* L. are cauline, ramel, opposite, exstipulate, simple, sub-sessile, ovate, entire, acute, unicostate, reticulate, rough, coriaceous and hairy. The stem is erect, herbaceous, quadrangular, branched, solid, green and pubescent. A tap and branched root system is present.

### Microscopical features

Vein islet No., vein termination No., palisade ratio, stomatal No., and stomatal index are given as in Table 1.

### Powder drug study

Trichomes of various types that is, multicellular, shagay, uniciniate, uniseriate etc (a-d), glandular hairs (e-f), tracheids (l), parenchyma cells (j), mesophyll cells (i), fragments of small veins (h), starch granules (g), fragments of epidermis and stomata (k) were found in the powder drug. Organoleptic evaluation of the powder drug is as seen in Figure 1.

### Phytochemical screening tests

Alkaloid, saponin, lignin, ca-oxalate, starch, protein, mucilage, tannin were identified in the plant extract. (Table 2).

### Hypoglycemic effect

The hypoglycemic effects of ethanolic extracts of *A. aspera* L. was compared with standard (glibenclamide). Both standard and ethanolic plant extracts reduced the blood glucose level of rabbits significantly ($P < 0.05$). The result showed that ethanolic plant extracts of *A. aspera* L. possess strong hypoglycemic effects. Higher doses of plant extract is more effective in decreasing blood glucose level when compared to low doses. The efficacy of low doses last for 2 to 3 h while high doses last for more than 3 h. (Table 3).

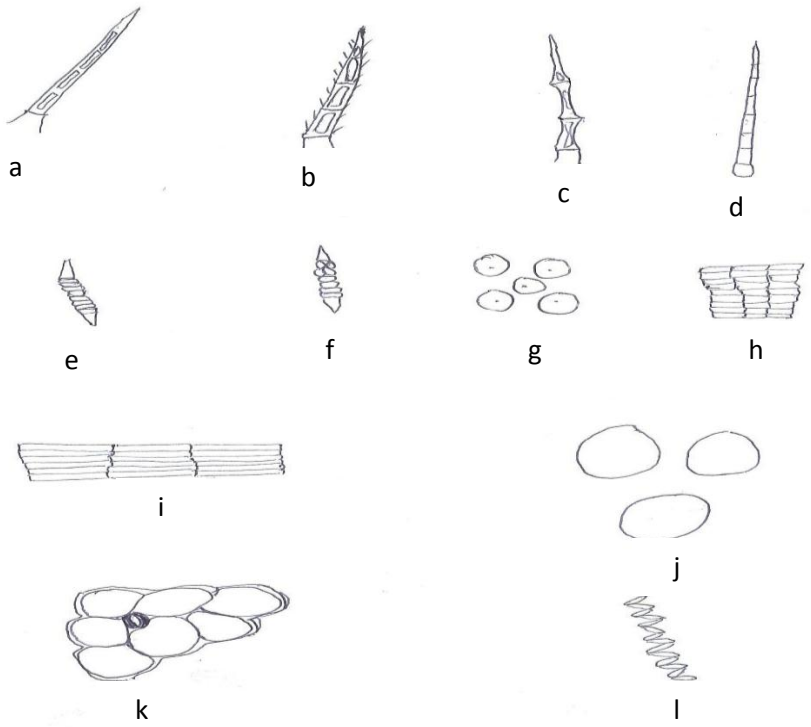


**Figure 1.** Powder drug study of the *A. aspera.* Multicellular (a), shagy (b), uniciniate (c), uniseriate (d), glandular hairs (e-f), starch granules (g), fragments of small veins (h), mesophyll cells (i), parenchyma cells (j), fragments of epidermis and stomata (k) and tracheids (l).

**Table 1.** Organoleptic evaluation of *A. aspera* leaves.

| S/No | Parameter | Observation |
|---|---|---|
| 1 | Color | Dark green |
| 2 | Odour | Pungent |
| 3 | Taste | Slightly disagreeable |
| 4 | Fracture | Fibrous |
| 5 | Touch | Soapy |

## DISCUSSION

The pharmacognostic profile is essential for crude drugs as it helps in the identification of specific drugs within the same genus. There are several plants within the same genus with similar structure and with different microscopic characteristics, however the medicinal value of these morphological similar plants vary from each other. Pharmacognostic studies help in the identification of specific plants possessing organoleptic and microscopic characteristics (Ismail et al., 2011).

Glibenclamide is a sulfonylurea derivative and commonly used in the management of diabetes mellitus type II; it causes hypoglycemia by stimulating beta cells of pancreas and increasing release of insulin and inhibiting glucagon secretion. As these effects require a functional pancreas, it can lower blood sugar levels in non-diabetic subjects (Soon and Tan, 2002) and a similarity was obtained in our tested ethanolic extract. It is very interesting to note that the ethanolic extract of the leaves of our selected plant is used in the tradition system of medicines in the management of various ailments. Our results show that the extract can decrease the serum glucose level in normoglycemic individuals. However, an adverse effect is that the plant can decrease the normal glucose level, just like glibenclamide, therefore, it is recommended that in the use of this plant as an anti-inflammatory or even for diabetic management, the glucose level should be monitored periodically. It is also worth mentioning that the plant should not be used in high doses for the treatment of ailments other than diabetes because it lowers the normal serum glucose level in normal patients.

In diabetes, oxidative stress has been found to be mainly due to an increased production of oxygen free radicals and a sharp reduction of anti-oxidant defenses (Oberley, 1988; Brownlee, 2001; Muhammad et al., 2012a). Hence, compounds with both hypoglycemic and anti-oxidative properties would be useful anti-diabetic agents (Brownlee, 2001). It has been reported that the methanolic extract of *A. aspera* is a good antidiabetic and antioxidant (Akhtar and Iqbal, 1991; Edwin et al., 2008) agent. The phytochemical study revealed the presence of

**Table 2.** Phytochemical screening of the EEA.

| Phytochemicals | Result | Remarks |
|---|---|---|
| Alkaloid | Reddish brown | ++ |
| Saponin | Frothing | ++ |
| Lignin | Pink coloration | ++ |
| Ca-oxalate | Dissolve in HCl without effervescence | ++ |
| Starch | Deep blue coloration | ++ |
| Protein | Yellow ppt. | ++ |
| Mucilage | Gummy suspension | ++ |
| Tannin | Greenish coloration | ++ |

**Table 3.** Effect EEA on blood glucose level of normoglycemic rabbits.

| Treatments (mg/kg) | 0 h | 1 h | 2 h | 3 h | 4 h | 5 h |
|---|---|---|---|---|---|---|
| Control | 153±2 | 151±1.5 | 152±2 | 149±2 | 147.7±2.5 | 142.3±1.15 |
| Glb (0.2) | 153.7±1.5 | 111.3±2.1** | 113.6±1.5** | 117.3±2.5** | 120.3±1.5** | 122.7±1.5** |
| EEA (100) | 149.7±1.5 | 114.3±2.1** | 118±2** | 123.6±2.5** | 133.7±3 | 139±2.6 |
| EEA (150) | 144±2 | 109.7±2.5** | 127.7±2.1** | 131.3±2.1** | 133±2 | 135.3±2.1 |
| EEA (200) | 150.7±2.5 | 90.6±2.1** | 101. 0±1.5** | 111.3±2.5** | 116.7±1.5** | 124.7±1.5** |

Glb= Glibenclamide, Data presented as mean ± SEM (n = 6). *P < 0.05, **P < 0.01.

starch which produce hyperglycemia but the presence of alkaloids, saponin and tannin in our results as hypoglycemic. Besides *A. aspera* L, many other plants like *Averrhoa bilimbi* (Pushparaj et al., 2000) and *Indigofera pulchra* (Tanko et al., 2008) have hypoglycemic effects on blood glucose level of normoglcemic rats. While plants like *Nauclea latifolia* (Gidado et al., 2005), *Rhinacanthus nasutus* (Rao and Naidu, 2010) and *Veranonia amygdalina* (Michael et al., 2010) show hypoglycemic effects in alloxin and streptozocin induced diabetic rats but have no pronounced effects on blood glucose in normoglycemic rats.

## Conclusion

The ethanolic extract of the leaves of *A. aspera* validates its use as a hypoglycemic drug. It can be used safely for the treatment of diabetes type two, however in certain cases, use of the drug by non-diabetic patient in high doses will lower the normal glucose level. It is therefore recommended that the plant should not be used in large doses for the treatment of non-diabetic conditions.

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*Full Length Research Paper*

# Nitric oxide-dependent vasodilation and intracellular $Ca^{2+}$ concentration increase induced by 6,8-dihydroxy-4'-methoxyflavone in rat aorta

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**In this study, we attempted to evaluate the role of endothelium-derived hyperpolarizing factor (EDHF) in the vasorelaxant response induced by 6,8-Dihydroxy-4'-methoxyflavone (DMF) in isolated rat aorta rings by using functional and biochemical approaches. Thoracic aortic rings were isolated and suspended in organ baths, and the effects of DMF were studied by means of isometric tension recording experiments. Nitric oxide (NO) was detected by ozone-induced chemiluminescence. The technique used to evaluate changes in intracellular ($[Ca^{2+}]_i$) in intact endothelium was determined by open aortic ring and loaded with 16 µmol Fura-2/AM for 60 min at room temperature, washed and fixed by small pins with the luminal face up. *In situ* electrical conductivities (ECs) were visualized by an upright epifluorescence Axiolab microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Zeiss × 63 Achroplan objective (water immersion, 2.0 mm working distance, 0.9 numerical apertures). ECs were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. In aortic rings with intact endothelium pre-contracted with norepinephrine (NE) ($10^{-4}$ M), the addition of DMF ($10^{-8}$ to $10^{-4}$ M) induced vasorelaxation in a concentration-dependent manner; in endothelium-denuded rings, the relaxant response induced by DMF was almost completely abolished suggesting that vasorelaxation was endothelium-dependent. DMF ($10^{-4}$ M) was able to significantly increase NOx levels. This effect was completely abolished after removal of the vascular endothelium. DMF (100 µM) caused a slow, long-lasting increase in $[Ca^{2+}]_i$. These results further support the hypothesis that DMF can induce activation of the NO/sGC/cGMP pathway, as suggested by functional studies. The results of the present study, using combined functional and biochemical *in vitro* approaches, indicated that DMF relaxes pre-contracted isolated rat aortic rings. Such a vasorelaxation was an endothelium-dependent effect, via the NO/sGC/cGMP pathway. This result also suggests that DMF causes a slow influx of extracellular $Ca^{2+}$. Release from the intracellular $Ca^{2+}$ stores and an inhibition of $Ca^{2+}$ extruding mechanisms.**

**Key words:** 6,8-dihydroxy-4'-méthoxyflavone, aorta, nitric oxide (NO), $Ca^{2+}$ signalling, endothelium, NO–cGMP pathway, vasodilation.

## INTRODUCTION

Some isolated compounds have been reported to mediate a plethora of relevant biological activities,

especially those related to vascular tone control. The bark parts of several species of Rutaceae, mainly of the genus Vepris, are considered aromatic and medicinal (Ngassoum et al., 2007; Hamawa et al., 2010). Phytochemical investigation of the stem barks of *Vepris heterophylla* (Engl.) R. Let. (Rutaceae), a medicinal plant used empirically in Cameroon by traditional healers in the treatment of various illnesses such as cardio-vascular disorders, especially arterial hypertension, and renal disorders, led to the isolation of the flavonoid, 6,8-dihydroxy-4'-methoxyflavone (DMF). To our knowledge, there have been no reports on the biological activity of this molecule. Flavonoids exhibit diverse biological effects, including inhibition of protein kinase C, inhibition of cyclic nucleotide phosphodiesterase, decrease in $Ca^{2+}$ uptake, and vasodilators actions. For the purpose of using 6,8-dihydroxy-4'-methoxyflavone, which remains as an unused resource after extraction from the bark of the trunk of *V. heterophylla* (Rutaceae) plant use as a medicinal therapeutic to treat hypertension and renal disorders (Toshiaki et al., 2006).

Endothelial cells play an important role in the control of vascular tone through the expression of constitutive or induced molecules on their surfaces, and the synthesis and secretion of soluble mediators are responsible for vascular homeostasis (Widlansky et al., 2003). Endothelial cells respond to a variety of neurohumoral and physical stimuli to release endothelium-dependent vasodilators such as endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980; Furchgott and Vanhoutte, 1989), prostacyclin (PGI2) (Jaffe, 1985; Vanhoutte et al., 1986) and endothelium-derived hyperpolarizing factor (EDHF) (Beny and Brunet, 1988; Feletou and Vanhoutte, 1988; Taylor and Weston, 1988; Nakane et al., 1991).

It is generally accepted that the release of EDRF, PGI2 and EDHF may have important physiological roles as dilator mediators in certain vessels. Other important mediators for relaxing the vascular smooth muscle are cyclic nucleotides (Murad, 1986). It is recognized that nitrovasodilators exert their effects on vascular smooth muscle by activating guanylate cyclase and increasing cyclic guanosine monophosphate (GMP), and that P-adrenoceptor agonists act by stimulating adenylate cyclase and increasing cyclic adenosine monophosphate (AMP). NO is derived from the oxidation of L-arginine by $Ca^{2+}$-calmodulin–dependent nitric oxide synthase (NOS), an enzyme constitutively expressed in the endothelial cells (Bredt et al., 1992; Yi-Ching et al., 2005). Furthermore, NOS can be activated by an Akt/PKB, $Ca^{2+}$-independent pathway (Arnold et al., 1997; Lowenstein et In this study, we attempted to evaluate the role of endothelium-derived factors in the vasorelaxant response induced by DMF in isolated rat aorta rings by using functional and biochemical approaches.

## MATERIALS AND METHODS

### Animals

Wistar rats (250 to 350 g) were used for all experiments. Animals were housed under conditions of controlled temperature (20 to 24°C) and humidity (55 ± 10%). In addition, they had free access to food (Harlan Teklad, Global diets, Pavia, Italia) and tap water *ad libitum*.

The animal handling was under the control of the veterinary surgeon of the University of Pavia. Experimental protocols and procedures were approved by the institutional Animals Care and Use Committee and the research was approved by the Ethical Committee of the University of Pavia.

### Drug administration

The following drugs were used: ATP, 6,8-dihydroxy-4'-methoxyflavone, Lantan 3+, dimethylsulfoxide (DMSO), norepinephrine, acetylcholine chloride, atropine sulfate, Nω-nitro-L-arginine methyl esther (L-NAME), indomethacin, L-arginine, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), all from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions were prepared in distilled water and kept at -4°C. DMF was solubilised in distilled water and diluted to the desired concentrations with distilled water just before use. ODQ was dissolved in DMSO. The other compounds were dissolved in distilled water. The final concentration of DMSO in the bath never exceeded 0.1%, and has no effect when tested in control preparations (data not shown).

### Chemical solutions

The composition of the Tyrode's solution used was 158.3 mM NaCl, 4.0 mM KCl, 2.0 mM $CaCl_2$, 1.05 mM $MgCl_2$, 0.42 mM $NaH_2PO_4$, 10.0 mM $NaHCO_3$, and 5.6 mM glucose. The physiological salt solution (PSS) had the following composition (in mM) 150 NaCl, 6 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Hepes, pH 7.4. In $Ca^{2+}$-free solution, $Ca^{2+}$ was substituted with 2 mM NaCl, and 0.5 mM EGTA was added. Both solutions were titrated to pH 7.4 with NaOH. Fura-2/AM was obtained from Molecular Probes (Molecular Probes Europe BV, Leiden, The Netherlands). All other chemicals were purchased from Sigma. Medium exchange and administration of agonists or other drugs were performed by removing the bathing medium (2 ml) and adding the desired solution. The medium could be exchanged quickly without producing artefacts in the fluorescence signal because a small meniscus of liquid remained between the tip of the objective and the facing surface of the cover slip.

### Plant collection and preparation of the extract

The stem bark was collected in February, 2010 in the Mokolo (Far North Region) in Cameroon (10° 39.214' N, 14° 24.145' E, 375 m of traditional uses. A sample was identified at the National Herbarium Cameroon (NHC) where a voucher specimen is deposited. The stem bark was cut air-dried and crushed. 2.5 kg of powder of the plant material was introduced into the extraction solvent at room temperature. The extraction lasted for 48 h. After decantation and filtration, the macerate was collected in a altitude). This region has annual average humidity of 73% and an average temperature of 29°C. This plant was selected on the basis volumetric flask. The

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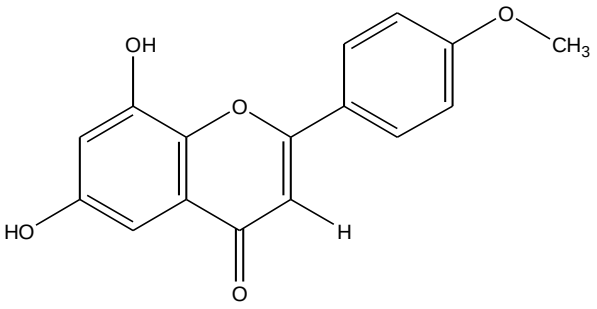


**Figure 1.** 6,8-Dihydroxy-4'-methoxyflavone structure.

operation was repeated two times with the solvent. Each extract was concentrated to dryness under reduced pressure using a rotary evaporator (BÜCHI). From this procedure, 175 g of ethyl acetate extract were obtained and kept at -0.5°C until use.

**Preparation of fractions and isolation of the molecule**

Fifty grams of the organic extract were subjected to flash chromatographic fractionation on a silica gel column (1.5 m length) of 40 ml eluted with hexane followed by gradient mixtures of hexane-ethyl acetate-methanol. Fractions of approximately 150 ml were collected and grouped on the basis of thin layered chromatography (TLC) on aluminum plate. Purification of the crystals obtained in the series of fractions was done by washing a solvent mixture of EtOAc-Hex (25/75) and preparative TLC, the compound 6,8-dihydroxy-4 '-methoxyflavone was obtained.

**Spectral characterization of DMF**

The use of spectral techniques: Nuclear magnetic resonance (NMR) to one or two dimensions, mass spectrometry (MS), UV-Visible spectroscopy and high performance liquid chromatography (HPLC) allowed us to clarify and determine the structure of the secondary metabolite isolated from the extract with ethyl acetate bark of the trunk of *V. heterophylla* (Rutaceae). This compound crystallizes as yellow flakes in the system Hex/AcOEt (30/70). Its UV spectrum shows respectively the wavelength λ = 248.7 nm (band II, cinnamoyl cycle), λ = 300 nm (band I, benzoyl ring) and the absorbance A = 2.625, A = 2.445 consider leaving structure flavonoid type. Its formula $C_{16}H_{12}O_5$ with 11 instaurations was deduced from its mass spectrum (TOF MS ES$^+$ 2.26 e4) in a positive ionization mode that shows pseudomolecule ions at m/z 286.14 $[M+H]^+$, 593.22 $[2M+Na]^+$; 878.29 $[3M+Na]^+$; 1163.49 $[4M+Na]^+$ involving its molecular weight 284 u. The $^{13}C$ NMR spectrum shows a mass of 18 carbon signals including a carbonyl at 180.77 ppm, eleven aromatic carbons in the range 86.68 to 159.30 ppm; a signal at 55.99 ppm characteristic of the methoxy group. The $^1H$ NMR spectrum shows five protons of aromatic type including 1 to 6.23 ppm is typical of H-2 protons of flavones, a signal to 3.72 ppm characteristic of the methoxy group; a signal at 8.45 ppm characteristic of the hydroxyl groups in position 6 and 8. The analysis of 2D COSY spectrum shows correlations between the signal at 7.85 and 7.23, 7.23 and 7.23, 7.85 and 7.85 ppm. The heteronuclear multiple-bond correlation spectroscopy (HMBC) spectrum allowed us to highlight the correlations between 7.85 and 126.57 ppm and 141.96, 115.57 and 7.23 ppm. The heteronuclear single quantum coherence (HSQC) spectrum shows the correlations 7.85 and 130 ppm, 7.23 and 114.57 ppm, 7.23 and 121 ppm. Spectroscopic data described in the literature are consistent with the values of the chemical shifts of the compound 6,8-dihydroxy-4 '-methoxyflavone (Gomes et al., 1983) (Figure 1).

**Preparation of isolated rat superior aorta rings**

Wistar rats were sacrificed by stunning and bleed. The thoracic and abdominal aorta were dissected out and perfused with Physiological Salt Solution (PSS). The superior aortic artery was removed and cleaned from connective tissue and fat. Rings (0.5 mm) were obtained and placed in Fura-2/AM for 1 hour; the rings were removed and stored in PSS for 30 min, at room temperature (22 to 24°C). When appropriate, the endothelium was removed by gently rubbing the intimal surface of the vessels. Rings (1 to 2 mm) were obtained and placed in physiological or Tyrode's solution, maintained to 37°C, and gassed with carbogenic mixture (95% $O_2$ and 5% $CO_2$) and maintained at pH 7.4. The preparations were stabilized under a resting tension of 1 g for 1 h; during this time, the solution was changed each 15 min to prevent the accumulation of metabolites (Flesch et al., 1998). The force of isometric contractions was recorded by a force transducer (Miobath-4; WPI, Sarasota, FL, USA) coupled to an amplifier-recorder (Miobath-4, WPI) and to a personal computer equipped with an analogue to digital converter board. The presence of functional endothelium was assessed by the ability of acetylcholine (10 µM) to induce more than 90% relaxation of vessels pre-contracted with norepinephrine (10 µM) and the absence, less than 10%, of relaxation to acetylcholine was taken as an evidence that the vessel segments were functionally denuded of endothelium (Dimo et al., 2005; Ntchapda et al., 2009). Vessels with intact functional endothelium and endothelium-denuded vessels showed no significant difference in the magnitude of contraction. The preparations were exposed to L-NAME (100 µM), a nitric oxide synthase (NOS) inhibitor (Moncada et al., 1993); L-NAME (100 µM) plus L-arginine (1 mM), the endogenous substrate of NOS (Toda et al., 2003); ODQ (10 µM), a soluble guanylyl cyclase (sGC) inhibitor (Garthwaite et al., 1995); or indomethacin (10 µM), a cyclo-oxygenase (COX) inhibitor (Moncada et al., 1991), plus atropine (1 nM), a non-selective muscarinic-receptor antagonist (Shiraki et al., 2001), used separately. These inhibitors were added 30 min before the application of norepinephrine. In the tonic phase of the second contraction, DMF ($10^{-7}$ to $10^{-4}$ M) was cumulatively added to the preparations. Inhibition was calculated by comparing the response of DMF before and after the addition of the inhibitor or antagonist.

**Determination of NO level**

The rat aorta was removed as described above, and rings 1 to 2 mm in width were placed in a 12-hole plate containing Tyrode's solution (0.7 ml/hole) and placed at 37°C for 40 min. Following incubation with the drugs, the middle of each well was collected and used for the determination of Nox and the rings weighed. The total amount of NOx in the medium was determined using the purge system of Sievers instruments (model NOA 280i, Boulder, CO, USA) (Braman et al., 1989). A saturated solution of vanadium chloride (VCl 3) in 1 M HCl was added to the nitrogen-bubbled purge vessel fitted with a cold water condenser and a water jacket to allow heating of the reagent to 90°C using a circulating bath. HCl vapors were removed by a gas bubbler containing sodium hydroxide (1 M). Flow of gas into the detector was controlled by a needle valve adjusted to yield a constant pressure. When the detector signal is stabilized, samples were injected into the purge ring to react with the reagent, thereby converting Nox to NO, which was then detected by ozone-induced chemiluminescence. NOx concentrations were calculated by comparison with a standard solution of sodium nitrate. Values for the control of the baseline were obtained from the aorta ring before the administration of

drugs. Rings (with or without the vascular endothelium) were then incubated with norepinephrine (10 µM) for 20 min. After this period, in each hole, 10 µM of acetylcholine was administered as a positive control and the DMF as negative control. After half an hour, the center of each hole was recovered and used for the determination of NOx, as described. To investigate whether NO production was $Ca^{2+}$-dependent, DMF was applied in an extracellular $Ca^{2+}$-free solution and in an extracellular $Ca^{2+}$-free solution after depletion of intracellular $Ca^{2+}$-store by:

1. Applying $La^{3+}$ (100 µM) in $Ca^{2+}$-free solution for 30 min, and then washing the ring by extracellular $Ca^{2+}$-free solution and applying DMF in extracellular $Ca^{2+}$-free solution.
2. Applying adenosine triphosphate (ATP) 300 µM in $Ca^{2+}$-free solution for 10 min, washing and incubating in $Ca^{2+}$-free solution for 20 min and then washing the ring by extracellular $Ca^{2+}$-free solution.

$Ca^{2+}$-free solution can be obtained by both avoiding $Ca^{2+}$ addition and adding EGTA [final concentration 0.5 mM (pH 7.4)]

#### Intracellular [$Ca^{2+}$] evaluation

The technique used to evaluate changes in intracellular ($[Ca^{2+}]_i$) in intact endothelium has previously been described (Moccia et al., 2002; Yuly et al., 2010). The aortic ring was opened and loaded with 16 µmol Fura-2/AM for 60 min at room temperature, washed and fixed by small pins with the luminal face up. *In situ* ECs were visualized by an upright epifluorescence Axiolab microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Zeiss × 63 Achroplan objective (water immersion, 2.0 mm working distance, 0.9 numerical apertures). ECs were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. The exciting filters were mounted on a filter wheel (Lambda 10, Sutter Instrument, Novato, Calif., USA). Custom software, working in the LINUX environment, was used to drive the camera (Extended-ISIS Camera, Photonic Science, Millham, UK) and the filter wheel, and to measure and plot online the fluorescence from 10 to 15 rectangular 'regions of interest' (ROI) enclosing one single cell. $[Ca^{2+}]_i$ was monitored by measuring, for each region of interest, the ratio of the mean fluorescence emitted at 510 nm when exciting alternatively at 340 and 380 nm (shortly termed 'ratio'). An increase in $[Ca^{2+}]_i$ caused an increase in the ratio. The experiments were performed at room temperature (21 to 23°C).

#### Data analysis

Mean values are presented together with standard error of the mean and the whole number of tested cells 'n' or number of experiments. Statistical significance ($p < 0.05$) was evaluated by the Student t-test and one-way analysis of variance (ANOVA), using Origin graph, (Microcal Origin 6.0) software version 6.0. Tracings shown in the figures are single cell recording. The potent vasorelaxation (pD2) value was calculated by non-linear regression. Emax is the maximal relaxation or maximal increase in $[Ca^{2+}]_i$ at the highest concentration used. The slope with correlation coefficient was measure by the Fit Linear regression. Values of $P < 0.05$ were considered statistically significant.

## RESULTS

### Relaxant activity of DMF

The mechanisms of DMF in causing vasorelaxation, and $[Ca^{2+}]_i$ increase in rat isolated thoracic aorta was investigated. In aortic rings with intact endothelium pre-contracted with norepinephrine ($10^{-4}$ M), the addition of DMF ($10^{-8}$ to $10^{-4}$ M) induced vasorelaxation [pD2 = 10.43 ± 3.29] in a concentration-dependent manner in endothelium-denuded rings, the relaxant response induced by DMF was almost completely abolished (pD2 = 1.08 ± 0.34) suggesting that vasorelaxation was endothelium-dependent (Figure 2). The maximal relaxant effects (Emax) of DMF in the presence and absence of the endothelium are reported in Table 1. In the presence of the NOS inhibitor L-NAME (100 µM), the relaxation induced by DMF in rings with or without endothelium was similar to that induced in endothelium-denuded rings (Figure 3). The inhibitory effect of L-NAME (100 µM) was completely reversed by the addition of the biological substrate of NO synthase L-arginine (1 mM) (pD2 = 09.93 ± 0.22) (Figure 3). In the presence of ODQ (10 µM), an inhibitor of the soluble guanylyl cyclase (sCG**)**, the relaxation induced by DMF ($10^{-8}$ to $10^{-4}$ M) was almost completely abolished (pD2 = 1.01 ± 0.32) (Figure 4 and Table 1). We also investigated the contribution of relaxant arachidonic acid derivatives and activation of the muscarinic receptors. In rings pre-incubated with indomethacin (1 µM) plus atropine (1 nM), the relaxation induced by DMF was not significantly different from the relaxation induced in endothelium-intact rings (pD2 = 11.16 ± 0.36) (Figure 4 and Table 1).

### NO production

In isolated rat aorta rings, DMF ($10^{-4}$M) was able to significantly increase NOx levels. This effect was completely abolished after removal of the vascular endothelium (Figure 5). Interestingly, when submitted to the same experimental conditions, acetylcholine ($10^{-5}$ M), used as positive control for NO production, induced an increase in NO- levels about 50% of that induced by $10^{-4}$ M DMF

### $Ca^{2+}$ signals

$Ca^{2+}$ signalling induced by DMF in *in situ* endothelium of aortic rings was also evaluated. DMF (100 µM) caused a slow, long-lasting increase in $[Ca^{2+}]_i$ (Figure 6). The amplitude of the $Ca^{2+}$ signal evoked by a high ATP concentration, known to be able to induce NO synthesis, was comparable with the increase in $[Ca^{2+}]_i$ evoked by DMF (Figure 7), suggesting that also DMF could be able to evoke a $Ca^{2+}$-dependent NO synthesis. In $Ca^{2+}$-free extracellular solution, the slow increase in $[Ca^{2+}]_i$ was still present, but with a slope (1.17, correlation coefficient = 0.95; n = 8) (Figure 8) much smaller than control values (8.31, correlation coefficient = 0.92; n=9), suggesting that $Ca^{2+}$ influx is involved. The introduction in the incubation medium of DMF 20 min before the higher magnitude response produced by ATP and 100 s before the addition

**Table 1.** Relaxation induced by DMF in the different experimental conditions.

| Experimental condition | Emax (% relaxation) | n |
|---|---|---|
| -Intact endothelium (E+) | 84.22±6.11 | 6 |
| -E+ plus L-NAME (100 µM) plus L-arginine (1000 µM) | 70.02±1.49 | 6 |
| -E+ plus L-NAME (100 µM) | 12.31±1.84*** | 6 |
| -E+ plus ODQ (10 µM) | 11.21±1.50*** | 6 |
| -Endothelium denuded | 18.49±2.52*** | 6 |
| -Endothelium denuded plus L-NAME (100 µM) | 16.75±1.94*** | 6 |
| -E+ indomethacin (1µM) plus atropine (1 nM) | 79.98±4.53 | 6 |

n, number of experiments. *** < 0.001 versus control.

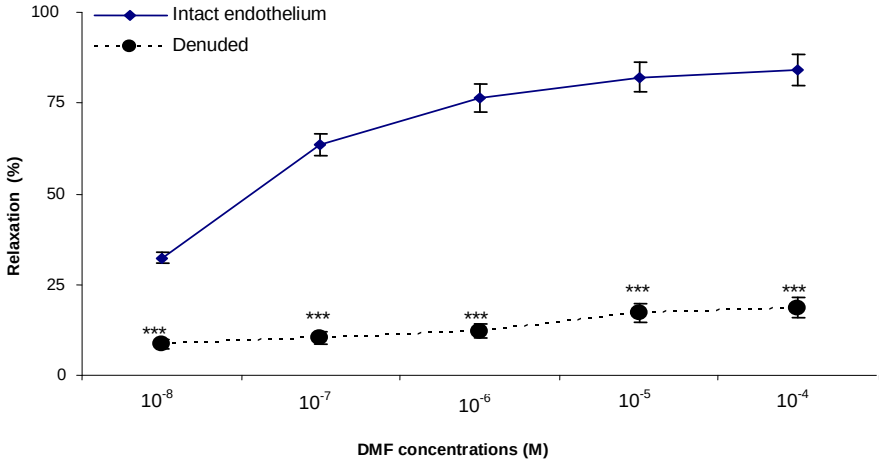


**Figure 2.** Relaxation induced by DMF in endothelium-intact (n = 6) or endothelium-denuded (n = 6) rat aortic rings. Values are mean ± SEM., *** P < 0.001.

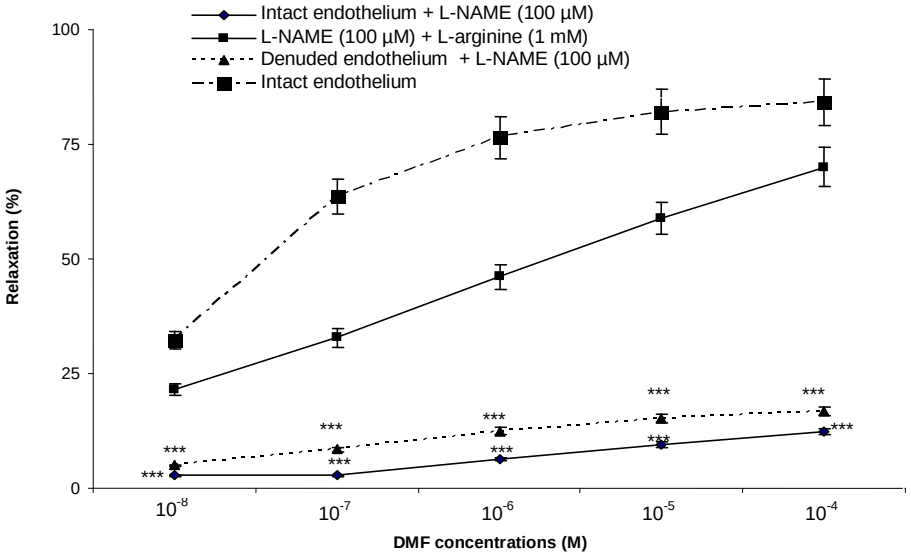


**Figure 3.** Relaxation induced by DMF ($10^{-8}$ to $10^{-4}$ M) in endothelium-intact rings (n = 6), endothelium-denuded rings, after pre-treatment with L-NAME (100 µM; n=6), and endothelium-intact rings after pre-treatment with L-NAME (100 µM) plus L-arginine (1 mM) (n = 6). Values are mean ± S.E.M., *** P < 0.001 versus endothelium-intact ring + L-NAME.

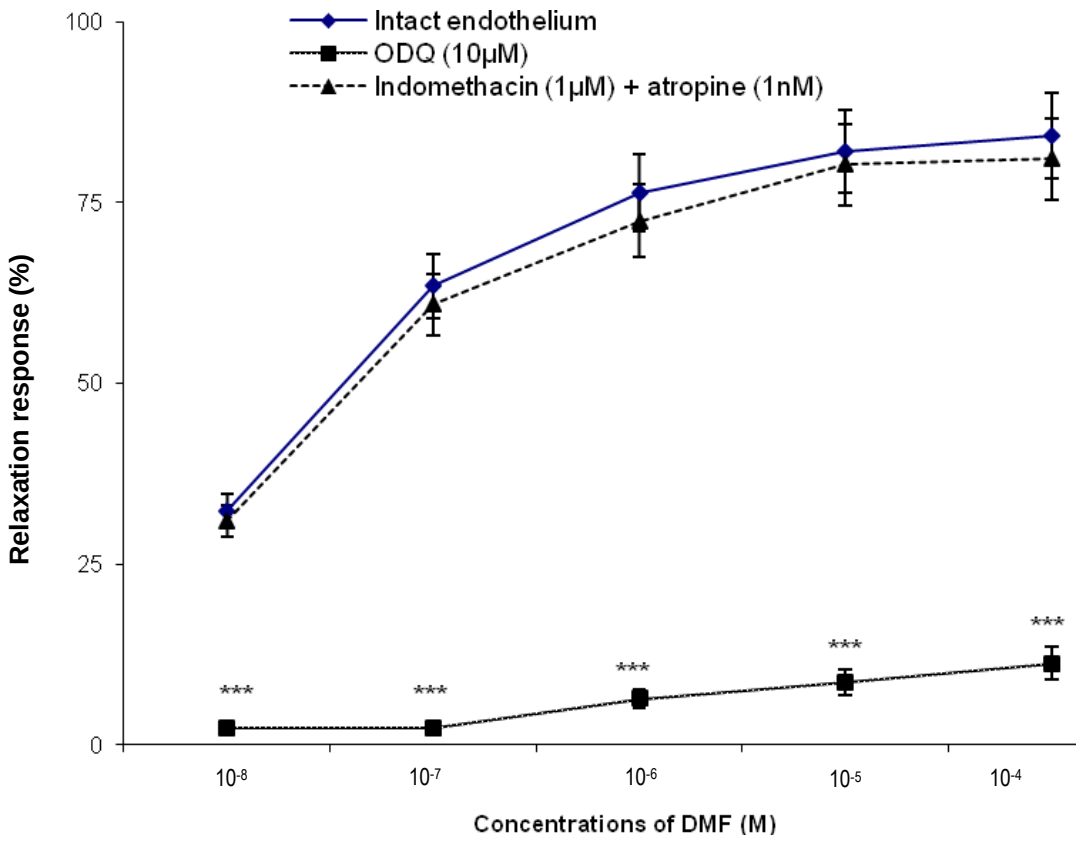


**Figure 4.** Relaxation induced by DMF ($10^{-8}$ to $10^{-4}$M) in endothelium-intact (n = 6) aortic rings, after (n = 6) pre-treatment with ODQ (10 µM), and after (n = 6) pre-treatment with indomethacin (1 µM) plus atropine (1 nM). Values are mean ± SEM. *** P < 0.001 versus endothelium-intact ring.

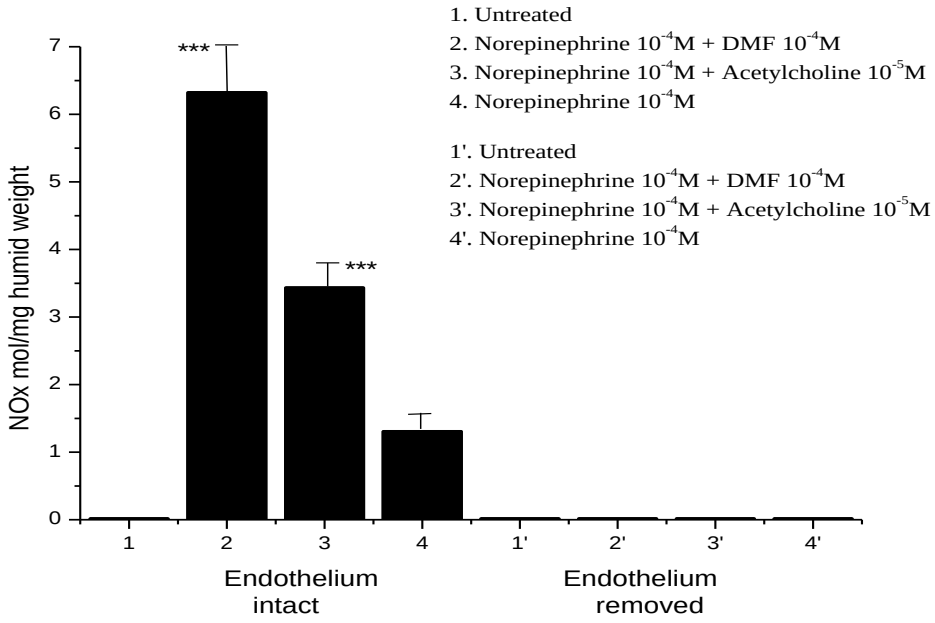


**Figure 5.** Effects of DMF ($10^{-4}$ M) and acetylcholine (ACh, $10^{-5}$ M) on NOx level in rat aorta rings pre-contracted with norepinephrine ($10^{-4}$ M), in aortic rings with intact endothelium (n = 5) or removed endothelium (n = 5). Mean ± SEM. *** P < 0.001 versus norepinephrine,

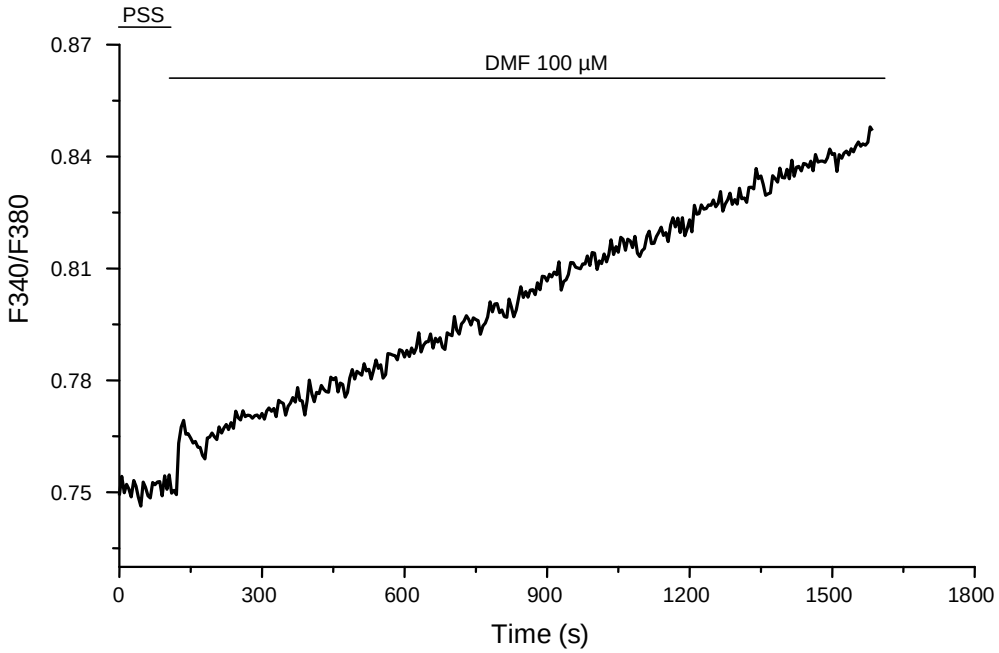


**Figure 6.** Effect of DMF (100 µM) on the intracellular $Ca^{2+}$ concentration of aorting ring endothelial cells (single cell tracing)

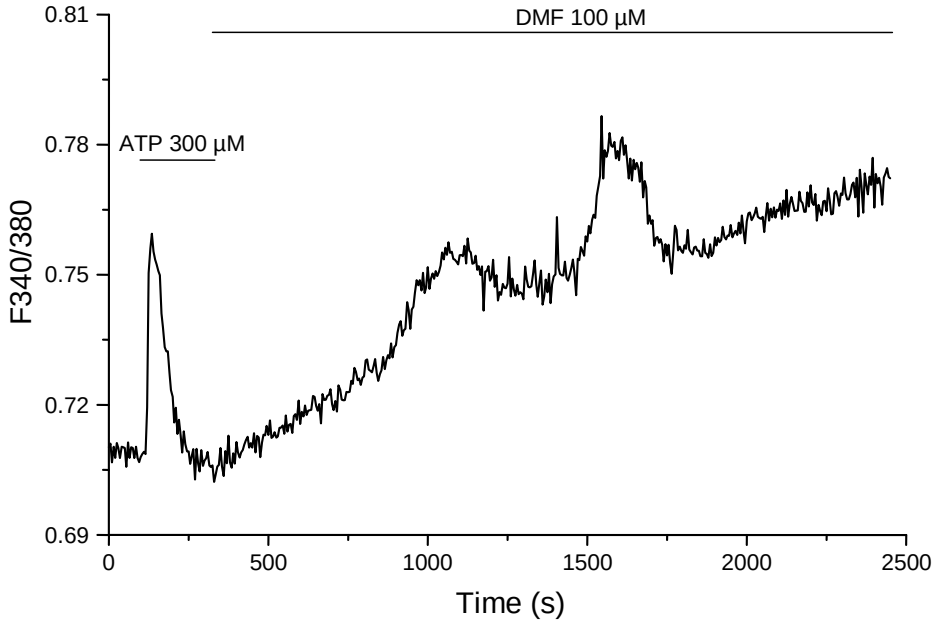


**Figure 7.** Effect of ATP (300 µM) and DMF (100 µM) on the intracellular $Ca^{2+}$ concentration of aorting ring endothelial cells (single cell tracing).

of DMF did not provoke any significant modifications of the amplitude effects of DMF (data no shown). In the presence of $La^{3+}$, the higher slope due to DMF (7.93, correlation coefficient = 0.97; n = 8) was completely abolished as shown in Figure 9, suggesting that DMF-induced intracellular influx $Ca^{2+}$ involves the participation of channels calcium. These results further support the hypothesis that DMF can induce activation of the NO/sGC/cGMP pathway, as suggested by functional studies.

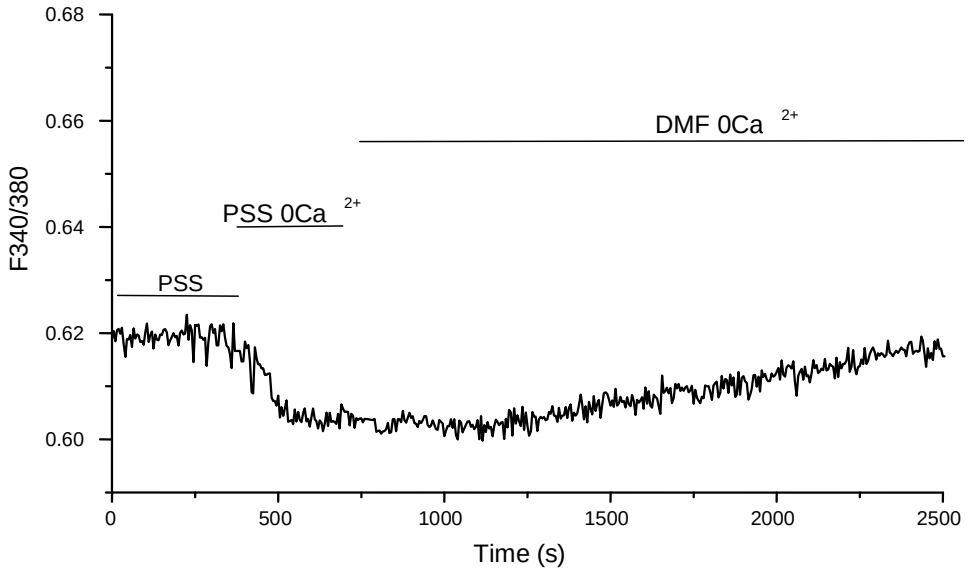


**Figure 8.** Effect of DMF (100 µM) on the intracellular $Ca^{2+}$ concentration of aorting ring endothelial cells in the presence (PSS) and absence of extracellular $Ca^{2+}$ ($0Ca^{2+}$) (single cell tracing).

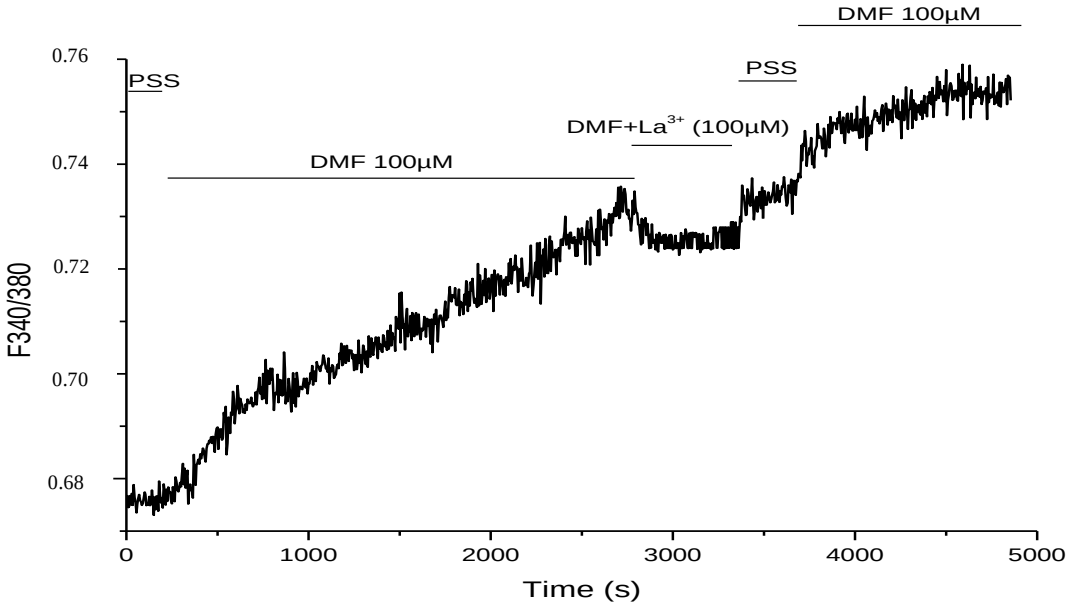


**Figure 9.** Effect of DMF (100 µM) on responses to the influx of extracellular $Ca^{2+}$ after (n = 8) pre-treatment of the rings with $La^{3+}$ (100 µM).

## DISCUSSION

In this paper, we have shown that DMF, a flavonoid isolated from stem bark of *V. heterophylla* induces a strong, dose-dependent relaxation of the rat aortic rings pre-contracted with norepinephrine. Furthermore, DMF relaxing activity is fully dependent on endothelial cells via the NO-cGMP pathway. Indeed, mechanical removal of endothelium abolished the relaxant response induced by DMF, suggesting that DMF activates an endothelium-dependent mechanism (Table 1 and Figure 2). To elucidate a mechanism, we first evidenced that inhibition of NO synthesis by the L-arginine analogue L-NAME abolished the vasorelaxation induced by DMF. Furthermore, in rings pre-incubated with L-NAME plus L-arginine, the vasorelaxante response by DMF was

completely reversed (Table 1 and Figure 3), suggesting that the DMF effect is mediated by a mechanism involving endothelium-derived NO. The action of NO, as a vasodilator, is mediated by the activation of vascular smooth muscle sGC, an enzyme that forms the second messenger cGMP, which activates a cGMP-dependent protein kinase (PKG) (Arnold et al., 1997; Lowenstein et al., 1994). To determine if DMF-induced relaxation involves the participation of a cGMP pathway, the preparations were pre-treated with ODQ, a soluble guanylyl cyclase inhibitor (Garthwaite, 1995). In these conditions, the vasorelaxation induced by DMF was almost completely abolished (Figure 4).

We also excluded that the endothelium-dependent vasorelaxant response may also involve the release of COX-derived products, such as PGI2, via the cyclo-oxygenase pathway (Furchgott and Zawadzki, 1980; Rapoport, 1986; Archer, 1994) or muscarinic receptor activation. To investigate the participation of theses pathways, we pre-treated the vessels with indomethacin (10 µM) plus atropine (1 nM). In this condition, DMF's effects were similar to that obtained in control conditions, suggesting that muscarinic-receptor activation or COX-derived products are not involved (Figure 4).

Taken together, these data provide strong evidence that DMF induces potent endothelium-dependent relaxations involving NO release. To further confirm such a hypothesis, a biochemical assay using a very sensitive technique for NOx analysis was performed (Chen, 1988; Braman et al., 1989). Since the half-life of NO is very short (a few seconds), nitrate, nitrite, and other nitroso compounds (NOxs), which are stable metabolites of NO, are frequently measured to determine NO production (López-Ramos et al., 2005). In endothelium-intact aorta rings pre-contracted with norepinephrine, DMF ($10^{-4}$ M) and acetylcholine ($10^{-5}$ M, used as positive control) significantly increased NOx levels. As illustrated in Figure 5, in response to $10^{-4}$ M DMF, NOx levels increased to virtually the same level as that reached after $10^{-5}$ M acetylcholine. Inversely, in rings in which the vascular endothelium was removed, no effect of DMF on NOx levels was observed, excluding a possible NO-donating effect induced by the compound.

It is well known that activation of constitutive endothelial NOS may be $Ca^{2+}$ dependent. DMF caused a slow, long lasting increase in the $[Ca^{2+}]_i$ of aortic endothelial cells. Such a slow $[Ca^{2+}]_i$ increase was very limited in $Ca^{2+}$-free extracellular medium (the slope in $Ca^{2+}$-free extracellular medium was only 14% of the slope in normal solution). This result suggests DMF causes a slow influx of extracellular $Ca^{2+}$ release from the intracellular $Ca^{2+}$ stores and an inhibition of $Ca^{2+}$ extruding mechanisms. $Ca^{2+}$ is known as an important regulatory element for many cellular processes; it acts either directly as a 2nd messenger or for the maximum activation of other enzymes in the signal cascade (Putney 1993; Berridge, 1997).

DMF ($10^{-4}$ M), administrated in $Ca^{2+}$ free medium did not increase intracellular influx $Ca^{2+}$. What suggest that DMF would act on the calcium channels by stimulating their openings, thus allowing a massive entry of $Ca^{2+}$ into the cell. To determine if DMF-induced intracellular influx $Ca^{2+}$ involves the participation of channels calcium, the preparations were pre-treated with $La^{3+}$ the non-specific calcium channel antagonists, which usually blocks calcium influx and calcium-related metabolic functions such as trans-membrane $Ca^{2+}$ transport within excitable tissues (Fitzpatrick, 1990), inhibited higher amplitude due to DMF-induced $Ca^{2+}$ release as illustrated in Figure 9. Negative effects of lanthanum on cells are attributed to its blockage of $Ca^{2+}$-channel on the cell membrane, and on this basis, a higher concentration of lanthanum (100 µM) has been extensively used to inhibit $Ca^{2+}$ influx to investigate various $Ca^{2+}$-dependent cellular processes in rat aorta (Jan et al., 1998; Lewis and Spalding, 1998; Geitmann and Cresti 1998; Friedman et al., 1998).

## Conclusion

The results of the present study, using combined functional and biochemical *in vitro* approaches, indicated that DMF relaxes pre-contracted isolated rat aortic rings. Such a vasorelaxation was an endothelium-dependent effect, via the NO/sGC/cGMP pathway. This result also suggests that DMF causes a slow influx of extracellular $Ca^{2+}$ release from the intracellular $Ca^{2+}$ stores and an inhibition of $Ca^{2+}$ extruding mechanisms.

## ACKNOWLEDGEMENT

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## ABBREVIATIONS

**DMF,** 6,8-Dihydroxy-4'-methoxyflavone; **NO,** nitric oxide; **$La^{3+}$,** lantan 3+; **ATP,** adenosine triphosphste; **pD2,** potent vasorelaxation; **DMSO,** dimethylsulfoxide; **L-NAME,** Nω-nitro-L-arginine methyl esther; **ODQ,** 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one; **$0Ca^{2+}$,** absence of extracellular $Ca^{2+}$ ; **$[Ca^{2+}]_i$,** intracellular $Ca^{2+}$ concentration; **PSS,** physiological salt solution; **HPLC,** high performance liquid chromatography; **NMR,** nuclear magnetic resonance.

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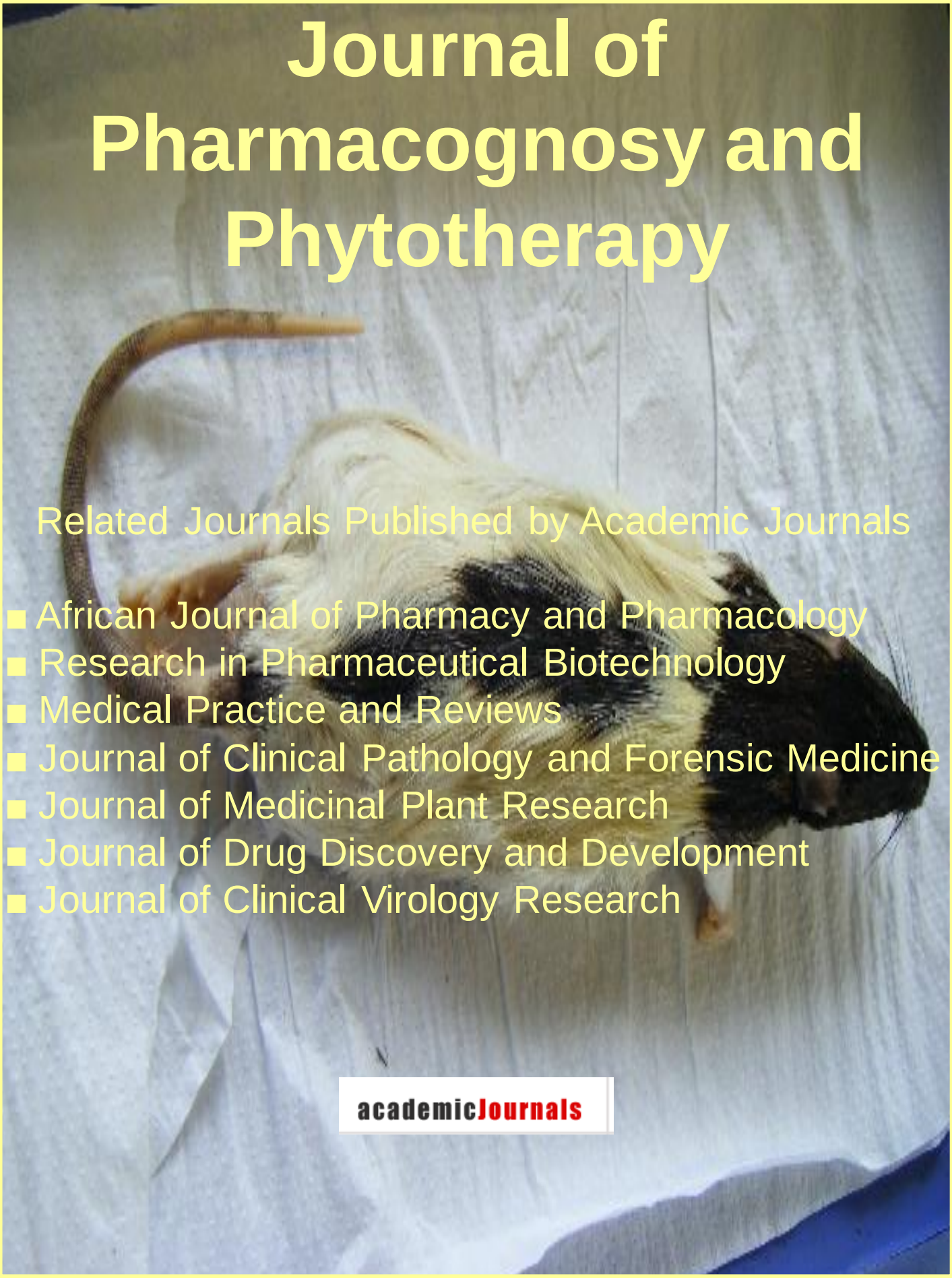

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