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# African Journal of Pharmacy and Pharmacology

Table of Contents: Volume 8, Number 2 15 January, 2014

## ARTICLES

### Research Articles

- Synergistic anticancer effects of valproic acid, atorvastatin and pioglitazoneoids in human malignant and murine cells** 31  
André L. S. Barros, Jaciana S. Aguiar, Larissa C. C. Araújo,  
Christina A. Peixoto, Paloma L. de Medeiros, Maria Teresa  
J. A. Catanho and Teresinha Gonçalves da Silva
- Could neuroplasticity be an answer to different antidepressants efficacy among individuals?** 40  
Z. H. Zheng, Y. T. Zeng, J. H. Tao, J. J. Duan, T. Zhou and F. Xu
- Antimicrobial and antioxidant evaluation of various parts of Cola milleni K. Schum plant** 46  
Orisakeye, O. T. and Ojo, A. A.
- An overview of ethnopharmacological properties of Boerhaavia diffusa** 49  
Humayun Riaz, Syed Atif Raza, Shahzad Hussain, Sidra Mahmood  
and Farnaz Malik
- Salman Iftikhar, Munazza Ahmad, Hina Muhammad Aslam, Tahir Saeed, palm pollen supplement** 58  
Salman Iftikhar, Munazza Ahmad, Hina Muhammad Aslam, Tahir Saeed,  
Yasir Arfat and Gul-E- Nazish

Full Length Research Paper

## Synergistic anticancer effects of valproic acid, atorvastatin and pioglitazone in human malignant and murine cells

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Valproic acid, atorvastatin and pioglitazone belong to different therapeutic drug classes and exhibit biological activities that are capable of interfering with cancer development. In this study, we examined the anticancer synergistic effects of these three drugs in experimental models *in vitro* and *in vivo*. Cytotoxic activity was determined against K562, NCI-H292 and HEp-2 lines cells. The Ehrlich carcinoma was used as *in vivo* model. Atorvastatin associated with pioglitazone, presented cytotoxicity against NCI-H292 cells with IC<sub>50</sub> value 3.75 µg/ml. The ultra-structural analysis showed that the atorvastatin in combination with pioglitazone induced apoptosis in 66.3% of the cells. Treatment with either valproic acid or valproic acid + atorvastatin + pioglitazone presented cytotoxicity in Ehrlich carcinoma cells, with IC<sub>50</sub> values equal to 10.8 and 11.4 µg/ml, respectively. In evaluation of antitumor effects *in vivo* it was observed that valproic acid or the atorvastatin + pioglitazone induced tumor inhibition of 60.2 and 64.9%, respectively. However, histopathology analysis suggests that the liver and kidneys are affected by both treatments. In conclusion, the data indicate that atorvastatin + pioglitazone present synergistic anticancer effect in lung cancer cells and solid tumours.

**Key words:** Anticancer, atorvastatin, Ehrlich carcinoma, thiazolidinedione.

### INTRODUCTION

Valproic acid (VPA), atorvastatin (ATOR) and pioglitazone (PIO) are drugs trade belonging to different therapeutic classes and are used widely in the world. The main indication for the use of VPA is the treatment of epileptic seizures, but it is also a histone deacetylase inhibitor (HDACi). Atorvastatin belongs to the class of statins, used mainly in the cholesterol control by inhibiting the 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA). Pioglitazone is a thiazolidinedione (TZD) with

hypoglycemic action. This class of molecules is peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) agonist. The potential of histone deacetylase inhibitors, statins and PPAR $\gamma$  agonists have been investigated as an alternative in treatment of various cancers (Marchion and Münster, 2007; Mrówka et al., 2008; Sassano and Plataniias, 2008).

Post-translational histone modifications such as acetylation are associated with transcriptionally active

regions of the genome. HDACs possess antitumor activities and have the potential to induce re-expression of genes abnormally suppressed in cancer cells, thus potentially inducing growth arrest, differentiation, and/or apoptotic cell death of transformed cells *in vitro* and *in vivo* (Bolden et al., 2006; Marks, 2010). Several HDACs are currently undergoing clinical testing and recently some have been approved for the treatment of cutaneous T-cell lymphoma (Munster et al., 2009). However, some research indicates that anticancer effects induced by these drugs may be more effectively exploited when used in combination with others chemotherapeutics (Ramalingam et al., 2007).

The anticancer effect of statins is the subject of numerous investigations, several results have reported efficacy of HMG-CoA reductase inhibitors against various types of cancer as leukemia, prostate, lung, colorectal and breast (Sławińska and Kandefer-Szerszeń, 2008; Bardou et al., 2010; Papadopoulos et al., 2011). PPAR $\gamma$  agonists demonstrate mechanisms of suppression of tumor development in several *in vitro* and *in vivo* models. Among the proposed mechanisms for the antitumor effects of TZDs, apoptosis induction, cell cycle arrest, and differentiation have been extensively reported (Grommes et al., 2006; Blanquicett, 2008; Lichter et al., 2008).

The concept of using a combination of agents for cancer chemoprevention or treatment has recently received much attention. Combinations of two or more agents that have different mechanisms of action have been suggested as a promising strategy to maximize efficacy and minimize toxicity (Lu et al., 2008). Although pre-clinical studies exist in the literature for ATOR, PIO and VPA, there are no reports of the anticancer effects induced by these drugs combined.

## MATERIALS AND METHODS

### Drugs and chemicals

Atorvastatin was obtained from LKT Laboratories, Inc., St. Paul, MN, USA. Valproic acid, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT), L-glutamine and doxorubicin were purchased from Sigma Aldrich Co., St Louis, MO, USA. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, penicillin (Pen) and streptomycin (Strep) were purchased from Gibco. Pioglitazone was obtained from Takeda Pharmaceuticals, Inc., Deerfield, IL, USA.

### Animals

A total number of 72 Swiss albino mice (male, 25 to 30 g), obtained from the animal house of Antibiotics Department of the Federal University of Pernambuco, Brazil, were used. The animals were kept in cages with free access to food and water, under 12 h light-dark cycles. The animals were treated in accordance with the International Council for Laboratory Animal Science (ICLAS), and following the ethical principles of the Brazilian Society of Science in Laboratory Animals (SBCAL). All experiments were approved by the Ethics Committee for Animal Experimentation of

the Biological Sciences Center of the Federal University of Pernambuco, Brazil, number 23076.002926/2009-06.

### Cells

The cytotoxicity of the drugs and drug combinations was tested against the following cell lines: K562 (human erythroleukemia), NCI-H292 (human lung mucoepidermoid carcinoma), HEp-2 (human laryngeal epidermoid carcinoma) and Ehrlich carcinoma (murine breast adenocarcinoma). The cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100  $\mu$ g/ml Strep and 100 U/ml Pen, and incubated at 37°C in a 5% CO $_2$  atmosphere.

Human cells were obtained from the Adolfo Lutz Institute, São Paulo, Brazil. Ehrlich carcinoma cells were maintained in the peritoneal cavities of the male swiss mice in the Bioassays Laboratory for Pharmaceutical Research, Antibiotics Department from the Federal University of Pernambuco.

### Cytotoxic activity

The cytotoxicity was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Mosmann, 1983). The tumor cell lines were seeded into 96-well plates (10 $^5$  cells/well for adherent cells or 0.5  $\times$  10 $^5$  cells/well for suspension cells; 100  $\mu$ l of medium). After 24 h, drugs or drug combinations (0.9 to 25  $\mu$ g/ml) were added to each well and incubated for another 24 h at 37°C in 5% CO $_2$ . Doxorubicin was used as positive control. After 72 h, 10  $\mu$ l of MTT solution (5 mg/ml) was added to each well and incubated for 3 h. At the end of this period, the medium was discarded and formazan crystals were dissolved in 100  $\mu$ l of DMSO. The absorbance of each sample was spectrophotometrically measured at 450 nm with a micro plate reader (Model 3550 BIO-RAD, Inc).

### Transmission electron microscopy (morphological detection of apoptosis)

For morphological assessment of apoptotic cell death, transmission electron microscopy was used for the NCI-H292 cell line, which showed the lowest IC $_{50}$  values for ATOR and ATOR + PIO. Cells were treated for 72 h and were later fixed with 2.5% glutaraldehyde (Sigma) and 4% paraformaldehyde (Sigma) in 0.1 M cacodylate (Sigma) buffer. After fixation, the samples were washed twice in the same buffer and post-fixed in 0.1 M cacodylate buffer (pH 7.2) containing 1% osmium tetroxide (Sigma), 2 mM calcium chloride and 0.8% potassium ferricyanide. Next, cells were dehydrated using acetone and embedded in SPIN-PON resin (Embed 812). Polymerization was performed at 60°C for three days. Ultrathin sections were collected on nickel 300-mesh grids, counterstained with 5% uranyl acetate and lead citrate and examined with an FEI Morgani 268D transmission electron microscope. A minimum of 40 cells per sample were observed from three independent experiments to evaluate any cellular morphological alterations (Ma et al., 2007).

### *In vivo* antitumor activity

Ehrlich ascites carcinoma (EAC) cells were derived from a spontaneous murine mammary adenocarcinoma. EAC cells were maintained in the undifferentiated form by passaging in syngeneic mice by transplanting 25  $\times$  10 $^6$  cells/ml (i.p.) each week. The ascitic fluid was removed by opening the belly and collecting all of the fluid with a sterile syringe. Ascitic tumor cell counts were carried out using the trypan blue dye exclusion method with a Neubauer

**Table 1.** Drug doses in mg/kg and drug combinations used for each experimental group.

Drug	Groups and doses (mg/kg)								
	1	2	3	4	5	6	7	8	9
NaCl 0.9%	-	-	-	-	-	-	-	-	-
Valproic acid	-	300	-	-	150	150	-	100	-
Atorvastatin	-	-	10	-	5	-	5	3.3	-
Pioglitazone	-	-	-	15	-	7.5	7.5	5	-
Doxorubicin	-	-	-	-	-	-	-	-	5

hemocytometer. Animals received 200  $\mu$ l of a suspension containing  $5 \times 10^6$  cells/ml (i.p.) as described previously (Matsuzaki et al., 2003). Treatment began 24 h after tumor cell inoculation and was administered once daily in a single dose for 7 consecutive days. In the treatments with isolated drugs, they were used in therapeutic doses, drug combinations were defined by dose response curve *in vitro*. The doses of each drug, drug combinations and treatment controls are listed in Table 1. For the negative control group, a 0.9% solution of NaCl was used. On the ninth day, the mice were sacrificed and the tumors were dissected, weighed and fixed in 10% formaldehyde. The inhibition rate (%) was calculated as follows: inhibition rate (%) =  $[(A - B) A^{-1}] \times 100$ , where A is the average weight of the negative control group and B is the average tumor weight of the treated group (Bezerra et al., 2008).

#### Toxicological analysis

Animals bearing tumors were observed daily throughout the experiment in order to identify any adverse reactions or abnormal signs. The body mass was measured at the beginning and end of the treatment regime. At the end of treatment, animals were anesthetized with ketamine and xylazine and blood samples were collected for hematological analysis. After sacrifice, the animals were autopsied to observe the internal organs with respect to position, form, size and color in order to describe signs of grave lesions. The kidneys, liver and spleen were removed, weighed and fixed in 10% formaldehyde.

Hematological analysis was carried out using an automatic cell counter (ABX-MICROS-60 cell counter Horiba, Inc). The samples were evaluated for the following hematological parameters: number of erythrocytes, concentration of hemoglobin, number of platelets and total count and differential of leukocytes.

#### Histopathological analyzes

After being fixed in formaldehyde, the tumors, livers, spleens and kidneys were put into paraffin. The blocks were cut using a microtome to a thickness of 4  $\mu$ m, and the slides were stained with hematoxylin and eosin for morphological analysis. Histological analysis was performed by light microscopy in order to recognize any alterations attributed to the drugs.

#### Calculations for the effects of drug combinations

The effects of drug combinations on the inhibition of cell growth were calculated using the combination index (CI), which is designed for drugs with "mutually nonexclusive" mechanisms of action (Chou, 1996; Reynolds and Maurer, 2005).

#### Statistical analysis

The results are presented as the mean  $\pm$  standard deviation (SD). The differences among the experimental groups were compared using an analysis of variance (ANOVA), followed by a Student Newman-Keuls test. The level of significance adopted in the tests was 5% ( $p < 0.05$ ).

## RESULTS

The cytotoxic activity of VPA, ATOR and PIO, alone and in various combinations, was tested against three human cancer cell lines as well as the murine Ehrlich carcinoma cell line (Table 2). ATOR and ATOR+PIO showed significant cytotoxicity against NCI-H292 cells with  $IC_{50}$  values of 5.54 and 3.75  $\mu$ g/ml, respectively. The CI for ATOR + PIO in this cell line was 0.57, indicating that this drug combination promotes synergistic effects.

No significant results were obtained from the cell lines HEP-2 or K562. VPA and ATOR + PIO showed significant cytotoxicity towards EAC cells with  $IC_{50}$  values of 10.8 and 11.4  $\mu$ g/ml, respectively. The CI value for the ATOR + PIO combination was 0.87, suggesting slight synergism. The doxorubicin positive control exhibited  $IC_{50}$  values statistically significant against all the cell lines studied.

Transmission electron microscopy is the best method for observing morphological changes in the cytoplasm and nucleus of cells and can delineate the apoptosis process with great precision. Figure 1A shows the typical morphology of control cells (treated with DMSO 0.1%) where  $88 \pm 3.7\%$  showed a complete surface of perfect clarity with the nuclear membrane and mitochondria in good shape. The ultrastructure of NCI-H292 cells treated with ATOR showed intense cytoplasmic vacuolization in  $43.4 \pm 6.3\%$  of the cells and  $26.2 \pm 4.8\%$  of chromatin condensation at the perinuclear margin (Figure 1B).

The cells treated with ATOR+PIO and doxorubicin showed typical apoptotic morphology in  $66.3 \pm 3.8$  and  $71.4 \pm 2.3\%$  of cells, respectively, beyond the stage of cytoplasmic and nuclear degeneration (Figure 1C and D).

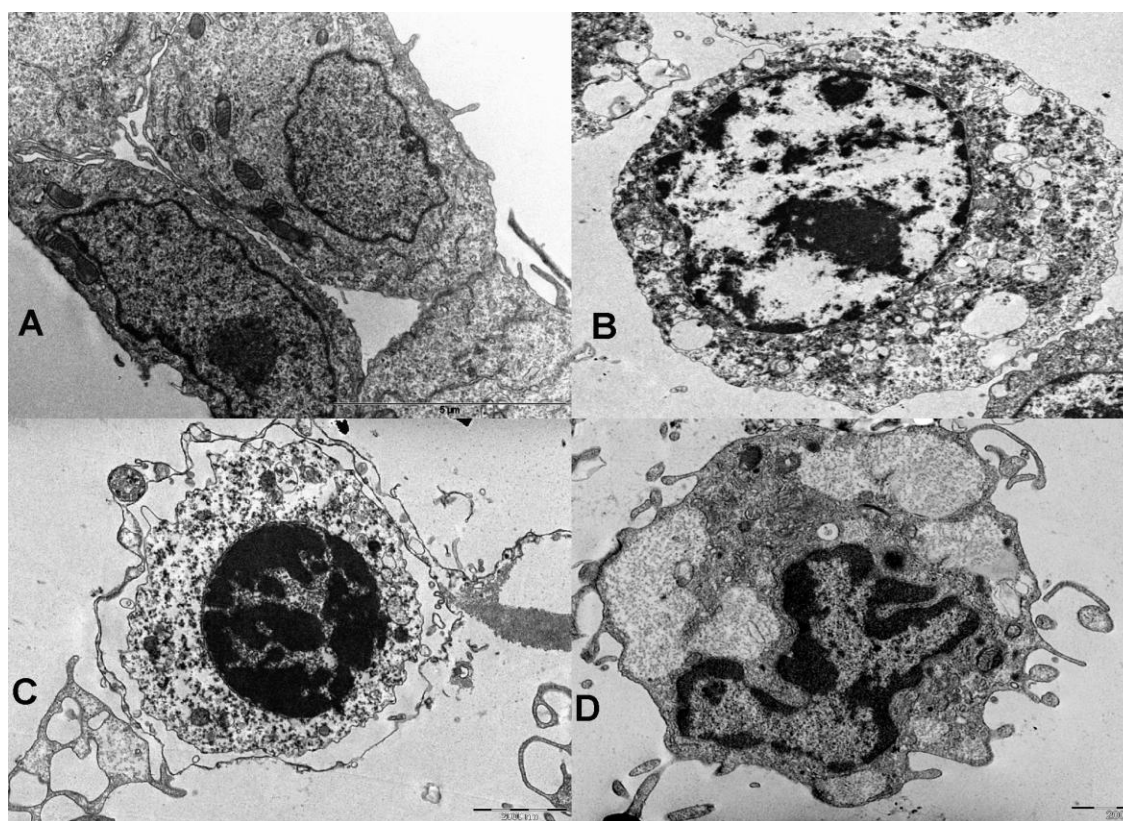
The effects of VPA, ATOR and PIO, alone and in various combinations, against Ehrlich carcinoma are described as shown in Figure 2. All of the treated groups showed tumor inhibition with respect to the control group



**Table 2.** The effect of individual drugs and drug combinations on human cancer cell lines and Ehrlich carcinoma.

Drug	IC <sub>50</sub> (µg/ml)			
	NCI-H292	HEp-2	K562	Ehrlich carcinoma
Valproic acid (VPA)	>25	>25	>25	10.8 (10.1 – 11.49)
Atorvastatin (ATOR)	5.54 (5.27 – 5.83)	>25	>25	24.79 (22.11 – 27.05)
Pioglitazone (PIO)	>25	>25	>25	>25
VPA + ATOR	15.26 (14.58 – 16.15)	>25	>25	23.6 (22.08 – 25.12)
VPA + PIO	>25	>25	>25	>25
ATOR + PIO	3.75 (3.57 – 3.92)	23.02 (22.12 – 24.26)	>25	11.39 (10.31 – 12.47)
VPA + ATOR + PIO	>25	>25	>25	>25
Doxorubicin	4.89 (3.98 – 5.69)	5.29 (4.12 – 6.31)	3.21 (2.93 – 3.99)	4.77 (2.33 – 7.41)

The data are presented as IC<sub>50</sub> values with 95% confidence interval from three independent experiments, carried out in quadruples.

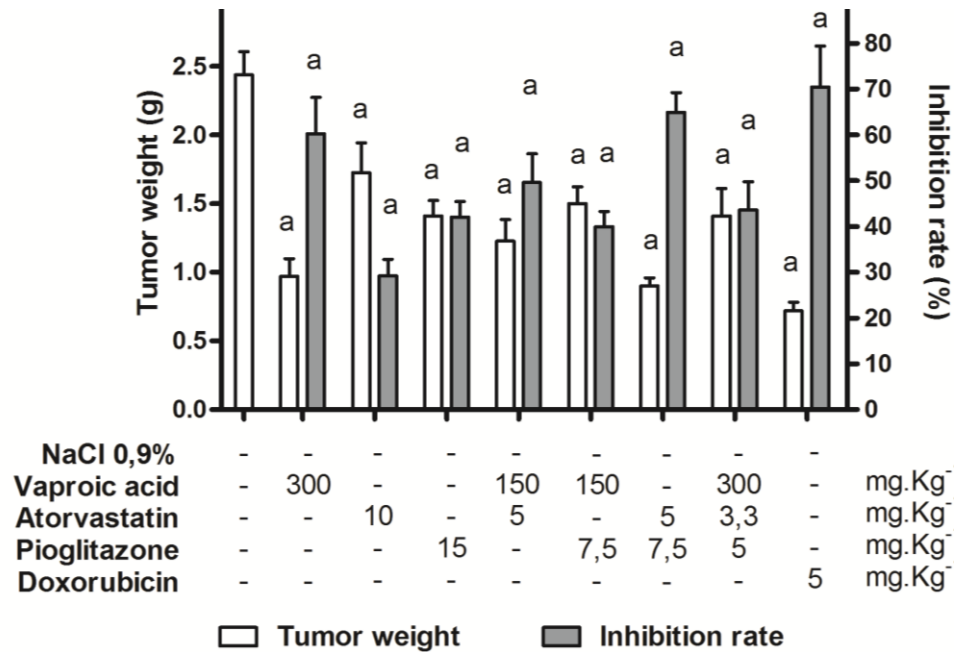


**Figure 1.** Transmission electron micrograph of NCI-H292 cells. (A) DMSO control (x5600): normal cell morphology; (B) ATOR (x7500): chromatin condensation at the perinuclear margin and many vacuoles; (C) ATOR + PIO (x7100): apoptotic cell with intense chromatin condensation and cytoplasmic degeneration; (D) Doxorubicin (x7100): apoptotic cell with complete degeneration of the nuclear membrane, chromatin condensation and cytoplasmic vacuoles.

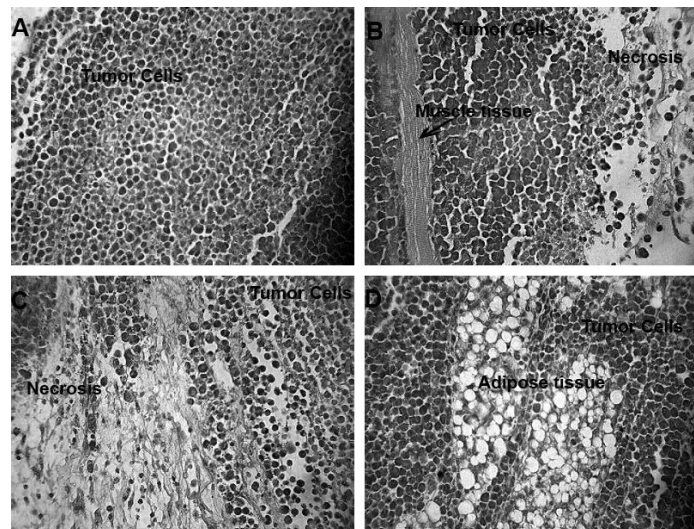
( $p < 0.05$ ). At the end of the experiment, the mean mass of the tumors from the control group was  $2.43 \pm 0.17$  g. In the animals treated with VPA, the weight of the Ehrlich carcinoma was reduced to  $0.96 \pm 0.13$  g; in the group treated with ATOR + PIO, the tumor mass was reduced to  $0.9 \pm 0.06$  g. The CI value for ATOR + PIO combination was 0.71, indicating a synergistic mechanism of action between the two drugs. In the group treated with

doxorubicin, the average tumor weight was reduced by 70.5%.

Histopathology analysis of the negative control tumors showed pleomorphic polygonal cells that were hyperchromatic and binucleated. Various degrees of nuclear and cellular pleomorphism were observed (Figure 3A). In the animal groups treated with VPA or ATOR + PIO muscular tissue invasion and extensive areas of coagulating necrosis



**Figure 2.** The effect of VPA, ATOR and PIO, alone and in combination, in mice transplanted with Ehrlich carcinoma. Values are presented as the mean  $\pm$  the standard deviation, where a = ( $p < 0.05$ ) compared with the control by ANOVA followed by the Student Newman-Keuls post-test.



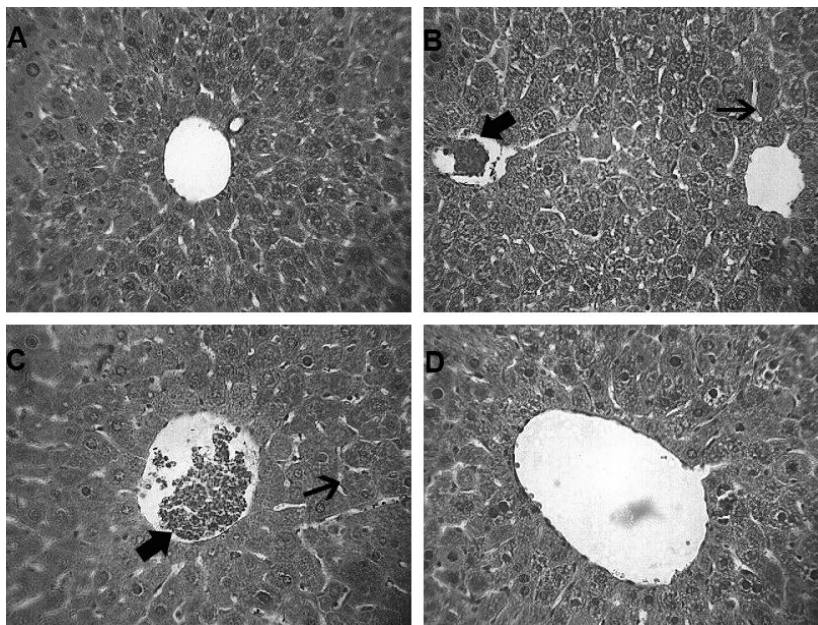
**Figure 3.** Photomicrograph showing the histopathology evaluation of the Ehrlich carcinoma from animals treated with 0.9% NaCl (A), 300 mg/kg VPA (B), 5 mg/kg ATOR + 7.5 mg/kg PIO (C) and 5 mg/kg doxorubicin (D) analyzed with a light microscope (x400).

necrosis were observed (Figure 3B and C). The group treated with doxorubicin showed invasion of adipose tissue (Figure 3D).

In the animals treated with VPA or ATOR+PIO, there was an increase in body weight; the mass gain was significantly different from the control and other groups ( $p <$

0.05). In the group treated with doxorubicin, there was a significant reduction of body mass with respect to the weight; these results were significantly different than the control ( $p < 0.05$ ).

Upon analyzing the blood from mice with Ehrlich carcinoma, statistically significant alterations were



**Figure 4.** The effect of AVP and the ATOR + PIO combination treatment on the liver of mice transplanted with Ehrlich carcinoma. Photomicrography showing the histopathology evaluation of the livers of animals treated with saline (0.9% NaCl) (A), 300 mg/kg VPA (B), 5 mg/kg ATOR + 7.5 mg/kg PIO (C) and 5 mg/kg doxorubicin (D) analyzed by light microscopy (x400). The thin arrow indicates the hydropic degeneration of hepatocytes and the large arrow indicates venous congestion.

those treated with VPA or doxorubicin (Table 3). Treatment with VPA induced greater thrombocytopenia versus the control and other experimental groups ( $p < 0.05$ ). Those animals treated with doxorubicin presented a reduction in the total number of platelets and leukocytes compared to the control and other treatment groups ( $p < 0.05$ ). The hematologic toxicity observed in this study are adverse reactions provoked by the drugs, mainly inhibiting bone marrow activity (Al-Harbi et al., 1992).

Histopathology analysis of the livers of animals in the negative control group presented well-preserved parenchyma, with morphologically regular hepatic cells constituting organized hepatic lobules. The hepatic sinusoids did not present abnormalities and the Kupffer cells appeared in normal quantities (Figure 4A). The animals treated with VPA and ATOR+PIO presented hydropic degeneration of hepatocytes and venous congestion (Figures 4B and 4C). The kidneys of the group treated with VPA did not reveal any relevant histological alterations compared with the negative control (Figure 5A and B). In the kidneys of animals treated with ATOR+PIO, some necrotic glomeruli and other signs indicative of partial necrosis of glomeruli were observed (Figure 5C).

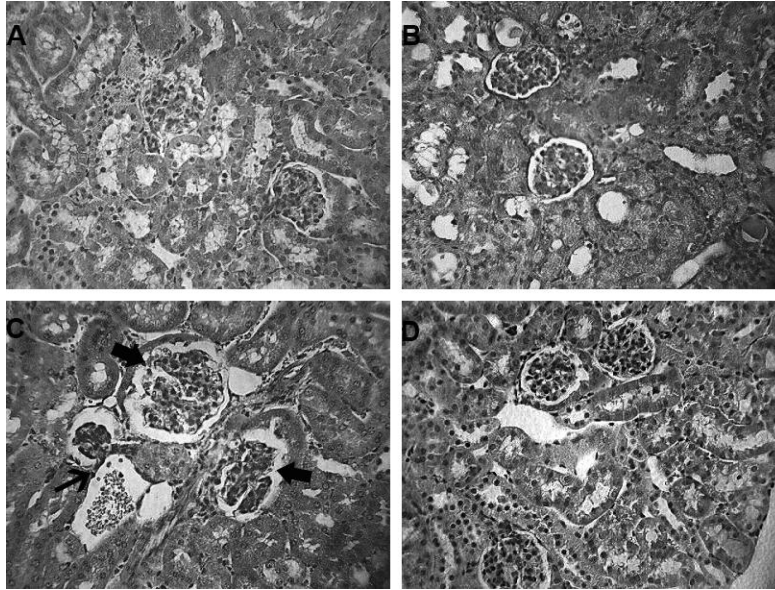
The spleens of the animals treated with VPA or ATOR+PIO did not show relevant histological alterations in relation to the negative control. The animals treated with doxorubicin (Figures 4D and 5D) did not present

relevant histopathology alterations in any of the organs evaluated in this study.

## DISCUSSION

This paper presents new results regarding the use of combinations of drugs that have different mechanisms of action. The synergistic effect of the ATOR+PIO combination stood out among our results; this combination induced apoptosis to cancer cell lines *in vitro* and revealed a prominent reduction in tumor growth *in vivo*.

In the human lung mucoepidermoid carcinoma cell line, treatment with ATOR or ATOR+PIO showed high cytotoxicity. The combination of ATOR+PIO produced a synergistic effect, inducing apoptosis in NCI-H292 cells. Moreover, the combination of these drugs displayed synergistic mechanisms of cytotoxicity *in vitro* and to Ehrlich tumor reduction *in vivo*. According Kaufman and Chabner (2001), drug combinations are especially effective when the additive or synergistic effects increase cytotoxicity for tumor cells while reducing adverse effects. Virtually all curative chemotherapy regimens employ multidrug combinations. However, the vast majority of those combinations have been developed empirically. Several studies have demonstrated the pro-apoptotic action of statins, which could relate to the decreased expression of anti-apoptotic proteins such as Bcl-2



**Figure 5.** The effect of VPA and the ATOR + PIO combination on the kidneys of mice transplanted with Ehrlich carcinoma. Photomicrography showing the histopathology evaluation of the kidneys from animals treated with 0.9% NaCl (A), 300 mg/kg VPA (B), 5 mg/kg ATOR + 7.5 mg/kg pioglitazone (C) and 5 mg/kg doxorubicin (D) analyzed by light microscopy (x400). The thin arrow indicates necrotic glomeruli and the large arrow indicates partial necrosis of the glomerulus.

(Dimitroulakos et al., 2000), the activation of caspases 3, 7, 8 and 9 (Cafforio et al., 2005), induced apoptosis via the suppression of ERK1/2 and Akt activation (Yanae et al., 2011) and the reactivation JNK, which is a kinase that can act as a tumor suppressor (Meral et al., 2007). Some pre-clinical studies have also shown that statins have a capacity to induce synergistic antitumor effects associated with conventional chemotherapeutics (Gan et al., 2008; Martirosyan et al., 2010; Chen et al., 2013).

PPAR $\gamma$  is ligand-dependent nuclear transcription factor that is highly expressed in adipocytes. PPAR $\gamma$  agonists have been demonstrated to inhibit cell proliferation and may act on cell cycle control check points (Koeffler, 2003; Heaney, 2004). The action of PPAR $\gamma$  is also related to apoptosis, principally by reducing the gene expression of BCL2 and increasing the expression of p27, c-myc, BAX and BAK (Ohta et al., 2001; Martelli et al., 2002; Li et al., 2003). Pioglitazone treatment has induced apoptosis and a notable decrease in cell proliferation in leukemia cell lines and in colon cancer cells (Zang et al., 2006; Cerbone et al., 2007).

The combination of ATOR + PIO or other statins and thiazolidinediones has shown synergic interactions towards the treatment and prevention of cardiovascular illnesses and diabetes through diverse experimental models (Forst et al., 2007, 2008; Hanefeld et al., 2007). Our results showed that ATOR+PIO inhibited proliferation of lung mucoepidermoid carcinoma; other studies also reported the anticancer effect of HMG-CoA inhibitor and

PPAR $\gamma$  agonist in lines of lung cancer (Li et al., 2010; Chen et al., 2012, 2013). Treatment with ATOR+PIO did not induce alterations in any measured hematological parameters, whereas histopathology analysis of the liver and kidneys revealed signs of toxicity, considered an adverse reaction knowingly associated with the metabolism of drugs used (Farley-Hills et al., 2004).

In this study, VPA induced considerable cytotoxicity *in vitro* and was able to inhibit the Ehrlich tumors *in vivo*. Our results corroborate those from other studies that demonstrate the effectiveness of VPA against diverse types of cancer. A reduction in tumor cell proliferation, an inhibition of angiogenesis, the promotion of apoptosis and provocation of cellular differentiation *in vitro* and *in vivo* have all been observed (Liu et al., 2006).

Several authors have adopted the hypothesis that VPA acts principally by inducing apoptosis and differentiation in various types of cancerous cells (Valentini et al., 2007). Experiments have demonstrated the efficacy of VPA in combination with other drugs in inducing apoptosis and in potentiating the activity of the combined drugs (Frew et al., 2008; Munster et al., 2009; Xie et al., 2012).

Our results revealed alterations in body mass, in hematological parameters and in hepatic toxicity induced by VPA. Devane (2003) attributes these physiological effects to the adverse reactions from VPA.

Conclusively, this study shows the promising potential of the drug combination ATOR + PIO. Until now, there have been no reports describing the therapeutic

**Table 3.** Effect of valproic acid, atorvastatin and pioglitazone, alone and in combination, on hematological parameters of the mice transplanted with Ehrlich carcinoma.

Parameter	Dose (mg/kg)	Redcells (10 <sup>6</sup> cells/ $\mu$ l)	Hemoglobin (g/dl)	Platelet (10 <sup>5</sup> cells/ $\mu$ l)	Total leukocytes (10 <sup>3</sup> cells/ $\mu$ l)	Differential leukocytes count (%)		
						Lymphocytes	Monocytes	Granulocytes
Saline (NaCl 0,9 %)	-	9.36 $\pm$ 0.53	11.3 $\pm$ 0.61	8.87 $\pm$ 0.71	8.13 $\pm$ 0.71	48.3	5.9	45.8
VPA	300	8.98 $\pm$ 0.42	12.35 $\pm$ 0.52	3.04 $\pm$ 0.53 <sup>a</sup>	8.62 $\pm$ 0.64	46.2	6.3	47.5
ATOR	10	9.17 $\pm$ 0.51	11.73 $\pm$ 0.39	8.65 $\pm$ 0.62	8.47 $\pm$ 0.67	41.1	8.2	50.7
PIO	15	9.82 $\pm$ 0.39	11.87 $\pm$ 0.54	8.57 $\pm$ 0.56	8.59 $\pm$ 0.63	43.7	7.2	49.1
VPA + ATOR	150 + 5	9.66 $\pm$ 0.47	12.94 $\pm$ 0.48	7.65 $\pm$ 0,57	8.09 $\pm$ 0.56	40.2	9.1	50.7
VPA + PIO	150 + 7.5	8.52 $\pm$ 0,52	12.41 $\pm$ 0.46	7.57 $\pm$ 0.52	8.25 $\pm$ 0.61	43,2	7.7	49.1
ATOR + PIO	5 + 7.5	9.53 $\pm$ 0.46	11.26 $\pm$ 0.38	7.84 $\pm$ 0.43	8.96 $\pm$ 0.69	45.1	6.4	48.5
VPA + ATOR + PIO	150 + 5 + 7.5	9.87 $\pm$ 0.59	10.53 $\pm$ 0.73	7.23 $\pm$ 0.51	7.95 $\pm$ 0.51	40.2	8.2	51.6
Doxorubicin	5	8.54 $\pm$ 0.86	11.14 $\pm$ 0.83	2.9 $\pm$ 0.47 <sup>a</sup>	2.19 $\pm$ 0.42 <sup>a</sup>	39.4	10.5	50.1

The values are presented as the average  $\pm$  the standard deviation from eight animals. <sup>a</sup>( $p < 0.05$ ) compared to the control and other groups by ANOVA followed by the Student Newman-Keuls post-test. Valproic acid (VPA); Atorvastatin (ATOR); Pioglitazone (PIO).

combination therapeutic combination of these drugs and their mechanisms of action. Our results reinforce the anticancer potential of valproic acid, atorvastatin and pioglitazone, demonstrating tumor growth inhibition *in vivo* with insignificant changes to the blood parameters or morphological and histopathological features.

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Review

## Could neuroplasticity be an answer to different antidepressants efficacy among individuals?

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Neuroplasticity is nervous system changes that occur in response to experience. Different individuals may have different neuroplasticity due to their different experiences. Even monozygotic twins may develop different neuroplasticity. Depression is a disorder of decreased neuroplasticity. Recent evidences suggest that antidepressants act by enhancing neuroplasticity, which allows environmental inputs to modify the neuronal networks to better fine tune the individual to the outside world. Meanwhile, there is a great individual difference of antidepressant response. So, it was concluded that variance of neuroplasticity in the depressant patients may play a role for individual difference of antidepressant efficacy.

**Key words:** Neuroplasticity, antidepressant, individual difference.

### INTRODUCTION

Neuroplasticity is nervous system changes that occur in response to experience. This idea was first proposed in 1890 by William James in *The Principles of Psychology*, though it was largely neglected for more than half a century (Berlucchi and Buchtel, 2009). It is well known that the brain consists of neurons and glial cells which are interconnected and all areas of the brain are plastic even after childhood (Rakic, 2002). For example, environmental changes could alter behavior and cognition by modifying connections between existing neurons and via neurogenesis in the hippocampus and other parts of the brain, like the cerebellum (Bonfanti et al., 2008; Bonfanti and Ponti, 2008; Ponti et al., 2008). It is evidenced that substantial changes occur in the cortical processing area, and that these changes can profoundly alter the pattern of neuronal activation in response to experience (Hanggi et al., 2011; Kiebel et al., 2008; Schlegel et al., 2012).

According to the theory of neuroplasticity, experience actually changes both the brain's physical structure (anatomy) and functional organization (physiology). Neuroplasticity is of importance in depression and

antidepressant treatment effects. Neuroscientists are presently engaged in a reconciliation of critical period studies demonstrating the immutability of the brain after development with the new findings on neuroplasticity, which reveal the mutability of both structural and functional aspects (Bakos et al., 2009; Farina et al., 2009; Goshen et al., 2009; Ilin and Richter-Levin, 2009; Kitanishi et al., 2009; Penn et al., 2009; Veyrac et al., 2009; Workman et al., 2009; Zhu et al., 2009). The paper discusses the potential role of neuroplasticity for difference of antidepressant efficacy.

### DIFFERENT INDIVIDUALS MAY HAVE DIFFERENT NEUROPLASTICITY DUE TO THEIR DIFFERENT EXPERIENCES

The development of central nervous system is affected by coactions of both genetics and environment. Enriched environment significantly improve the brain growth and brain damage repair (Mundinano and Martinez-Millan, 2010)

Prolonged stress can negatively impact capacity for neuroplasticity (Pittenger and Duman, 2008). A hypothesis was proposed that expression of neuroplasticity is a form of adaptation based on natural selection, where cells deprived of sensory input actively go and look for information in order to survive (De Ridder and Van de Heyning, 2007).

Different individuals have different degrees of neuroplasticity due to their different experiences (Zheng and Xu, 2012). It is widely accepted that environmental factor has an important role in this difference (Badenoch and Cox, 2010; Chung et al., 2009; Jarvenpaa et al., 2004; Siok et al., 2008). Furthermore, the estimated number of human protein-coding genes is around 35,000 (Ewing and Green, 2000). Meanwhile each hemisphere of human brain occupies about  $10^{11}$  neurons, let alone the hundreds of connections that each neuron makes. This suggested that human genes contain too little information to specify neural system and there must be an important random factor in neural development (Zheng and Xu, 2012). Cortical laminar development shows a process that is mathematically consistent with a random walk with drift (Landing et al., 2002). Cerebral cortex has a range of interconnected functional architectures. Some exhibits random and without structure, while others are geometrical (Siegel and Read, 2001). Additionally, epigenetic factors have a role in neural development, which will lead to different expressions of a gene (Fraga et al., 2005). Thus, even monozygotic twins may develop different neural structure and neuroplasticity, though they share the identical gene background. These are evidenced by discordance in some diseases morbidity as the following examples. An investigation showed that significant hippocampal atrophy was detected in the demented twins when compared with the controls. Meanwhile, in the non-demented twins, non-significant reduction was observed in the hippocampal volumes compared with the controls (Jarvenpaa et al., 2004). Some monozygotic twins are discordant in many diseases such as bulimia nervosa (Bulik et al., 2001), schizophrenia (Cannon et al., 2000), bipolar disorders (Gourovitch et al., 1999), and sexual orientation (Hall and Love, 2003).

## **DEPRESSION IS A DISORDER OF DECREASED NEUROPLASTICITY**

Various stress, such as bereavement, earthquake and disease (e.g. cancer), is a major environmental component for increased susceptibility to depression (Calabrese et al., 2011; Johansson et al., 2011; Liu et al., 2011; Morina et al., 2011). Depression is associated with a hyper-responsiveness to chronic stress (Hafner et al., 2011). Chronic stress, which can precipitate or exacerbate depression, decrease neuroplasticity, while antidepressant treatment produces opposing effects and can increase neuroplasticity.

Accumulating evidence demonstrates that neuroplasticity is decreased in depression. Though some researchers suggest that the complex biological and psychological changes associated with depression cannot be attributed to disturbance in hippocampal neurogenesis alone (Bessa et al., 2009; Tang et al., 2012), it is board acceptance that stress triggers activation of the hypothalamic-pituitary-adrenal (HPA) axis, and causes the brain to be exposed to corticosteroids, affecting neurobehavioral functions with a strong downregulation of hippocampal neurogenesis, and is an important risk factor for depression (Kubesova et al., 2012; Masi and Brovedani, 2011; Santarelli et al., 2003). Early-onset depression and risk for depression are associated with decreased neuroplasticity (Lopez et al., 2010).

As important products of HPA axis, glucocorticoids are stress-induced steroid hormones (Numakawa et al., 2010), and increased glucocorticoid level is correlated with depression (Howell et al., 2011). Early life stress can program the development of the HPA axis, causing alterations of neurochemistry and signaling pathways related to neuroplasticity regulation, and then changing neurobehavior (Lai and Huang, 2011). Chronic or severe stress and high-dose treatment with glucocorticoids is surely decrease of hippocampal synaptic plasticity and morphological neuroplasticity. Prolonged stress can negatively impact hippocampal function and capacity for neuroplasticity. Various forms of acute and chronic stress and elevated levels of glucocorticoids have been shown to reduce hippocampus neurogenesis. Chronic restraint stress induces significant regression of the apical dendrites of pyramidal cells in medial prefrontal cortex (mPFC) and negatively impact mPFC function (Pittenger and Duman, 2008).

Stress and depression lead to atrophy of hippocampus. Glia loss and neuronal abnormalities have been observed in the prefrontal cortex in major depression. Noradrenergic axons have been found with reduced axonal arborization and density after stress exposure. Serotonergic axon sprouting seems to be dependent on brain-derived neurotrophic factor (BDNF), one of the neurotrophin family and a critical cytokine of neuroplasticity, which appears to be decreased after stress exposure. Therefore, it appears that stress and depression may increase neuronal atrophy degeneration. Additionally, hippocampal neurons continue proliferation well into adulthood. This continued neurogenesis relies on the presence of BDNF and serotonin and inhibited by glucocorticoids (Gould, 1999; Lee et al., 2002), both of which are altered in depression. Increasing evidence shows that antidepressant treatment may reverse the atrophy of hippocampal neurons, increase cell survival, and enhance monoamine axonal sprouting (Rush et al., 2004).

Cognitive function depends on hippocampal plasticity (Vanguilder et al., 2012). Recent data imply that hippocampus-specific deletions of BDNF in rodent models



induce cognitive deficits as well as impairment in extinction of aversive memory (Heldt et al., 2007). Accordingly, these animal model data are consistent with theory that decreased neuroplasticity within the hippocampus could contribute to depression. Particularly, these data suggest that hippocampal plasticity deficits contribute to both the cognitive deficits and the inability to decrease negative affective and cognition that are the characteristic of major depression.

Thus, alterations in neuronal populations in the depressed state likely contribute to the dysregulated affective neural circuitry. Reduced neuronal density and atrophy in the hippocampus and prefrontal cortex presumably contribute to decreased activity responsiveness. In addition, these processes likely serve to maintain a state of imbalance in depression by reducing the ability of cortical and hippocampal areas to inhibit or modulate the stress pathways of the amygdale and interconnected circuitry. The depressed state is the one in which these stress pathways cannot be easily returned to normal, and the individual is left in a chronic state of abnormal affective responsiveness.

#### **ANTIDEPRESSANTS TARGET ON NEUROPLASTICITY**

Clinical and basic researches demonstrate that chronic antidepressant treatment increases the rate of neurogenesis in the adult hippocampus. Antidepressants up-regulate cAMP and the neurotrophin signaling pathways involved in plasticity and survival. *In vitro* and *in vivo* data provide direct evidence that the transcription factor, cAMP response element-binding protein (CREB) and BDNF are key mediators of the therapeutic response to antidepressants. Depression maybe associated with a disruption of mechanisms that govern cell survival and neuroplasticity in the brain (D'Sa and Duman, 2002).

Emerging research in experimental animals changed the perception of researchers to understand stress as well as the effects of antidepressant agents. Recent findings from the basic neurosciences to the pathophysiology of depression suggest that stress and antidepressants have reciprocal actions on neuronal growth and vulnerability (mediated by the expression of neurotrophin) and synaptic plasticity (mediated by excitatory amino acid neurotransmission) in the hippocampus and other brain structures. Stress has the capacity to progressively disrupt both the activities of individual cells and the operating characteristics of networks of neurons, while antidepressant treatments act to reverse such injurious effects (Reid and Stewart, 2001). Antidepressants increase the expression of several molecules, which are associated with neuroplasticity, in particular BDNF and its receptor TrkB. Antidepressants also increase neurogenesis and synaptic numbers in several brain areas. Fluoxetine, a selective

serotonin reuptake inhibitor (SSRI) antidepressant, can reactivate developmental-like neuroplasticity, which under appropriate environmental guidance, leads to the rewiring of a developmentally dysfunctional neural network (Balu et al., 2009; Guirado et al., 2009).

Antidepressants may act by enhancing neuroplasticity, which allows environmental inputs to modify the neuronal networks to better fine tune the individual to the outside world. Recent observations directly support this idea. According to the network hypothesis of depression, BDNF may act as critical tools in the process whereby environmental conditions guide neuronal networks to better adapt to the environment. Antidepressants may indirectly produce an antidepressant effect by increasing BDNF levels (Castren and Rantamaki, 2010).

#### **INDIVIDUAL DIFFERENCE IN ANTIDEPRESSANT EFFICACY**

Are antidepressants truly effective in all patients? Meta-analysis of all available trials of each antidepressant in the treatment of major depression, including treatment resistant depression and long-term relapse prevention is conducted by many researchers (Bauer et al., 2009; Kennedy et al., 2009; Rahimi et al., 2009). The efficacy and safety of antidepressants vary significantly. New evidences showed that the total effective rate of fluoxetine was about 77% (Duan et al., 2009). Various classes of antidepressant medications generally induce remission of major depression in only about one-third of patients. One double-blind study suggested the superiority of different combinations of antidepressants from treatment initiation. 105 patients meeting DSM-IV criteria for major depression were randomly assigned to receive, from treatment initiation, either fluoxetine monotherapy (20 mg/day) or mirtazapine (30 mg/day) in combination with fluoxetine (20 mg/day), venlafaxine (225 mg/day titrated in 14 days), or bupropion (150 mg/day) for 6 weeks. The primary outcome measure was the Hamilton Depression Rating Scale (HAM-D) score. The overall dropout rate was 15%, without notable differences among the four groups. Compared with fluoxetine monotherapy, all three combination groups had significantly greater improvements on the HAM-D. Remission rates (defined as a HAM-D score of 7 or less) were 25% for fluoxetine, 52% for mirtazapine plus fluoxetine, 58% for mirtazapine plus venlafaxine, and 46% for mirtazapine plus bupropion (Blier et al., 2010).

Although the use of antidepressants increased markedly during the 1990s, in recent years it has decreased as a result of concerns regarding the emergence of suicide during antidepressant treatment. There is evidence that SSRIs can improve adolescent depression better than placebo, although the magnitude of the antidepressant effect is 'small to moderate', because of a high placebo response, depending on the different

individual. A cautious and well-monitored use of antidepressant medications is a first-line treatment option in adolescents with moderate to severe depression. Low rates of remission with current treatment strategies indicate that further research in both psychotherapy and pharmacotherapy is warranted (Masi et al., 2010).

## CONCLUSIONS

Why antidepressant efficacy varies among individuals? The degree of neuroplasticity is different in depressed patients. An individual difference in relation to antidepressant efficacy is identified. Neuropsychiatric disease treatment among individuals who have different nervous system structure and function may produce different efficacy (Zheng and Xu, 2012). So, neuroplasticity might be an answer to different antidepressants efficacy among individuals. Although, evidences for this hypothesis are needed to strengthen this important mechanistic links between antidepressant efficacy and neuroplasticity, it is suggested that the individual difference in neuroplasticity may play a role for individual difference in antidepressant treatment response. This hypothesis can be tested by comparing the antidepressant efficacy among different BDNF levels or anatomical structures. Furthermore, since the nervous system is the leading system in the human body, neuroplasticity may also play role in other disease treatment efficacy.

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Full Length Research Paper

## Antimicrobial and antioxidant evaluation of various parts of *Cola milleni* K. Schum plant

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The methanol extracts of the various plant parts were screened for anti-oxidant activity by thin layer chromatography using 2,2-diphenyl-1-dipicrylhydrazyl (DPPH) while their *in vitro* antimicrobial evaluation were determined by agar diffusion method and bioautographic technique using *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* as test organisms; aqueous solvent served as control. The result showed that methanol extracts of the epicarp were found to be stronger against the entire test organisms except *E. coli* and were also found to possess strong antioxidant activity. The seed from the fruit, stem and root bark of the plant were not active against the entire test organisms. The antimicrobial property shown by the leaf is an evidence of the ethno medicinal uses of the plant. Phytochemical screening of the plant showed the presence of saponins, peptides and sugars.

**Key words:** *Cola milleni*, 2,2-diphenyl-1-dipicrylhydrazyl (DPPH), *Escherichia coli*, *Bacillus subtilis*, *staphylococcus aureus*, *Pseudomonas aeruginosa*, antimicrobial, antioxidant activity.

### INTRODUCTION

*Cola milleni* K. Schum is one of 125 species from the genus *Cola* Schott & Endl from the family Sterculiaceae (Ratsch, 2005). Sterculiaceae is a botanical name for a group of flowering plants at the rank of family, as is true for any botanical name, the circumscription, status and placement for the taxon varies with taxonomic point of view. The family name is based on the genus *Sterculia*. As traditionally circumscribed, the sterculiaceae, Malvaceae, Bombacaceae, and Tiliaceae comprise the "core Malvales" of the croquist system and the close relationship among these families are generally recognised (Watson and Dallwitz, 1992). The genus was formerly classified in the family Malvaceae subfamily Sterculioideae and was later transferred into the separate family sterculiaceae (Sonibare, 2009). *Cola* is one of the

most popular genera in the family of about 70 genera, totaling around 1,500 species of tropical trees and shrubs. It is related to the genus *Theobroma* which is also part of the family (Orisakeye and Olugbade, 2012).

*Cola milleni* K. Schum is a tree that grows to 12 m high, occasionally to 20 m with a low crown of arching branches; deciduous, of closed and transition forest, tending toward the drier parts, in Ivory Coast to Southern Nigeria (Adegoke et al., 1968). As a tree, it grows vigorously, and it has been successfully called monkey kola in English and Atewo-edun as Yoruba people would call it or achi okokoro (Ibo).

The bark has been reported to show alkaloids (Adegoke et al., 1968). Odugbemi (2006) reported that leaves of *C. milleni* are used in the treatment of ringworm,

ringworm, scabies, gonorrhoea, dysentery and ophthalmic. The fruit is bright red in a stellate cluster; its seed is covered with a felted fibrous coat. The kernel is edible. The wood is white and very resilient. It is used in Nigeria for the stock of the cross bow and in Liberia for rat traps and bows.

The antimicrobial property and its phytochemistry of the leaf ethanol extract of *C. milleni* was also done using human strain (Sonibare et al., 2009). According to Adeniyi et al. (2004), only the leaf and stem bark of *C. milleni* were reported not to be active at the highest concentration of 1000 microg/ml. Only the methanol extract root bark of *C. milleni* was found to be potent against both *Mycobacterium bovis* and strains of *Mycobacterium vaccae*. In spite of the popularity of the plant in traditional application previous phytochemical and antimicrobial studies have been limited to the leaf and stem bark. The present study examines the seed, epicarp, leaf, stem and root bark for their antioxidant and antimicrobial property.

## MATERIALS AND METHODS

### Plant

All the plant parts materials were collected from a tree located on Obafemi Awololowo University (OAU) campus (Road 1). It was authenticated by comparison with herbarium specimens by the Department of botany, OAU.

### Extraction of plant material

The fruits of *C. milleni* were separated into epicarp and seeds. The epicarp was then macerated (930 g) in methanol (100%) at room temperature for 72 h. The seed (1630 g), stem bark (1107 g), leaf (463 g) and root bark (450 g) were air-dried, powdered and extracted in aqueous methanol at room temperature for 48 h. The mixture of each extraction was then filtered using filter paper. The filtrate was concentrated to dryness *in vacuo* to yield crude extract *C. milleni* stem bark (CMSB) (23.06 g), *C. milleni* root bark (CMRB) (24.28 g), *C. milleni* leaf (CML) (31.13 g), *C. milleni* epicarp (CME) (12.13 g) and *C. milleni* seed (CMS) (15.34 g).

### Antioxidant screening

Crude extracts of all the parts of the plant were screened for anti oxidant activity. The brief description of the procedure is as follows: A solution of the test material in method was spotted on the thin layer chromatographic plate and developed using a suitable solvent system. This was sprayed with DPPH reagent. The chromatogram was exposed to daylight until the purple violet background was bleached. Only zones where the colour turned yellow within the first fifteen minutes after spraying were recorded as positive results (that is, possess antioxidant activity). The result of the screening of the different plant materials are summarized in Table 1.

### Antimicrobial screening

The agar diffusion (cup plate) method was used for this examination. Molten and cooled agar 60 ml (45°) were separately inoculated

with the nutrient broth culture of the test organisms (0.6 ml) and mixed thoroughly. The inoculated medium was then carefully poured into sterile petri dishes (24 cm petri dish) and allowed to set. Thereafter, cups (8 mm diameter) were aseptically bored into the solid nutrient agar using a sterile cork borer. The test solutions 100 ul each were then introduced into each of the cups ensuring that no spillage occurred. Also, the same volume of the standard antimicrobial agent and the solvent were introduced into some of the cups to act as positive and negative controls, respectively. The plates were left at room temperature for 2 h to allow for diffusion into the medium and thereafter incubated face upwards at 37°C for 24 h. Sample was tested in duplicate and diameters of zone of inhibition were measured to the nearest millimetre using transparent ruler (Onawumi, 1997).

### Bioautography technique for anti-microbial screening

The method involved an overlay of inoculated agar medium on developed silica gel thin layered chromatography (TLC) glass plate followed by incubation at 37°C for 24 h. Zones of inhibition were detected as clear white areas over a purple background (Onawumi, 1997).

### Phytochemical screening

The dried, pulverised leaves were subjected to phytochemical analysis to screen for peptides, cyclopeptides and sugars.

### Test for peptides

The extracts were examined for presence of peptides using TLC technique with solvent system dichloromethane: methanol: acetic acid (8:2:2) as the mobile phase. A reverse phase plate was the stationary phase while freshly prepared ninhydrin in acetone was the detecting agent. Presence of cyclopeptides was studied in the crude extract by developing two spotted silica plates (A and B) in solvent system dichloromethane: methanol: acetic acid (8:2:2). Sample on one of the plates (B) was hydrolyzed by heating the plate in a covered glass vessel containing concentrated hydrochloric acid at 100°C for 1 h. Both plates (A and B) were subsequently sprayed with ninhydrin in acetone. Any ninhydrin positive spot on B but absent in A is taken as positive for the presence of cyclopeptides.

### Test for sugars

A solution of the test material was spotted on the TLC plate along side with glucose, sucrose, fructose, lactose and developed using a suitable solvent system. This was sprayed with p-anisaldehyde-sulphuric acid.

### Test for phenolics using ferric chloride

It is used in the detection of phenolics. A solution of FeCl<sub>3</sub> (1 g) in methanol (40 ml) is prepared for spraying on TLC plates.

## RESULTS

The results of antioxidant tests and antimicrobial activities

**Table 1.** Antimicrobial activities of both the crude extract from *Sterculia tragacantha* and *Cola milleni* in 80mg/ml concentration in methanol:H<sub>2</sub>O (1:1).

Test organism	Diameter of zone of inhibition (mm)							
	STRB	CML	CME	CMS	STSB	CMRB	CMSB	Solvent
<i>Escherichia coli</i> NCTC 10418	-	-	-	-	-	-	-	-
<i>Staphylococcus aureus</i> NCTC 6571	5	-	15	-	5	-	-	-
<i>Pseudomonas aeruginosa</i> ATCC 10145	-	6.5	6	-	-	-	-	-
<i>Bacillus subtilis</i> NCTC 8236	6	6.5	10	-	7	-	-	-

Cup size = 8.0 mm, - = no activity.

**Table 2.** Antioxidant activities of the crude extracts of various parts of *Cola milleni*.

Sample	Source	Time taken for colour development (DPPH method)	Antioxidant activity
CML	Leaf	No reaction	-
CMS	Seed	Immediate	Strong
CMRB	Root bark	10 min	Weak
CMSB	Stem bark	10 min	Weak
CME	Epicarp	5 min	Strong

## Peptides

All the various parts of the plant was screened for peptides. Only *C. milleni* epicarp showed the presence of peptides.

## Cyclopeptides

Out of all the various parts of the plant screened for cyclopeptides, *C. milleni* stem bark showed the presence of cyclopeptides. Unlike the case of peptides, cyclopeptides are detectable with ninhydrin only after hydrolyses, since the detection involve reaction of ninhydrin with the free primary amino function. However, amides are expected to show similar characteristics as the cyclopeptides in the test system.

## Sugars

*C. milleni* epicarp showed the presence of fructose and *C. milleni* seed showed the presence of both the lactose and fructose. This would explain why the mucilagenous cover of the seed is sweet. On the other hand, no sugars were detected in the remaining parts of the plant.

## Phenolics

*C. milleni* root bark and epicarp showed the presence of phenols. On the other hand, no phenols were detected in the remaining parts of *C. milleni* examined.

## DISCUSSION

The crude extracts of the various parts of *C. milleni* showed reaction with DPPH after some minutes except the leaf. The CMS showed immediate colour reaction and it was a strong anti-oxidant activity. The stem and root barks showed a colour reaction after 10 min and it was a weak anti-oxidant activity. This is suggestive that further work could be done on it to know the active principle that is responsible for the activity. The CME possess strongly anti-oxidant activity after 5 min. This is also suggestive that if further work is done on it extensively the seed and the epicarp of the plant could serve as a better free radical scavenger and inhibitor of oxidative tissue damage than vitamin C, vitamin E succinate, vitamin C and vitamin E succinate combined, and beta carotene.

In this study, various parts of the crude extracts of *C. milleni* were subjected to preliminary antimicrobial test. *C. Milleni* leaves were active against gram-negative *Pseudomonas aeruginosa* at a minimum diameter of zone of inhibition of 6.5 mm and gram-positive *Bacillus subtilis* at a minimum diameter of zone of inhibition of 6.5 mm but not active against *Escherichia coli* and *Staphylococcus aureus*. Interestingly, the antimicrobial activity of the leaf was in line with the previous antimicrobial works of *C. milleni* (Sonibare et al., 2009; Adeniyi et al., 2004; Ebana et al., 199; Reid et al., 2005).

CME showed no activity with *E. coli* but were active against *S. aureus* at a minimum zone of inhibition of 15 mm, *P. aeruginosa* at a minimum zone of inhibition of 6 mm, *B. subtilis* at minimum zone of inhibition of 10 mm.

CMS, CMRB and CMSB did not show any activity with any of the test organisms. Hopefully, CMS, CMRB and CMSB are not wastes materials because of their antioxidant activities. Further work is being done by the author of this paper to isolate the active compounds responsible for both the antimicrobial and antioxidant activity each.

## Conclusion

The antimicrobial property of the leaf justifies its use in treating ringworm, scabies, dysentery, gonorrhoea and ophthalmic. Unfortunately, people lick the seed and throw away its epicarp. This research findings has shown that the epicarp possesses strong antimicrobial activity and also show a strong antioxidant activity implying that it could also be used to treat ringworm, scabies, dysentery to mention a few and could serve as a free radical scavenger.

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Review

## An overview of ethnopharmacological properties of *Boerhaavia diffusa*

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Herbal medicines are used by traditional healers since hundreds of years, and medicinal plants are imperative reserve for traditional and herbal industry. This practice is in vogue since ancient times. Over 80,000 species of plants are used globally for their medicinal uses. *Boerhaavia diffusa* is one of the distinguished medicinal plants used to treat large number of human ailments. The plant in whole or its parts (aerial parts and roots) have abundant medicinal properties and are used by common and ethnic people for their common ailments. The investigated and reported pharmacological and therapeutic properties of *B. diffusa* include laxative and immunomodulatory activities, cancer chemopreventive efficacy, inhibition of tumorigenesis, antioxidant activity, hepatoprotective activity, hypoglycemic activity, antifungal, anti-proliferative, anti-estrogenic, analgesic, potent anti-fibrinolytic, antibacterial activity, anticonvulsant activity, diuretic, spasmolytic activity, anti-stress and dyspepsia. It has been studied with keen interest due to its promising medicinal values in phytochemical and pharmacological research field. The objective of this review on *B. diffusa* is to sum up plant morphology, chemical compositions, ethno-medicinal uses with a scope of development in future. The manifold benefits of *B. diffusa* has made it a true miracle of nature.

**Key words:** *Boerhaavia diffusa*, antioxidant, hepatoprotective, hypoglycemic, antifungal, anti-proliferative, anti-estrogenic, analgesic, antibacterial.

### INTRODUCTION

*Boerhaavia* species has been studied extensively for its varied promisable therapeutic effects due to its phytochemical and pharmacological investigational grounds. Hermann Boerhaave, a famous Dutch physician of the 18th century invented this plants (Chopra, 1969). *Boerhaavia diffusa* L. is a species of developing plant in the four o'clock family (Nyctaginaceae) which is generally recognized as tar vine. It is a persistent herbaceous plant growing in moist areas such as the India, South America and Antilles Africa (Correˆa, 1984). Individuals who are native to most of the Australian zone would use it like a net to capture small birds. Scientific names for *B. diffusa*

are; *Boerhaavia repens* var. *diffusa*, Syn. *Boerhaavia repens* while family names are Horse Purslane, Hog weed. Various common Indian names used for *B. diffusa* are Mukaratee-Kirei (Tamil), Punernava (Telugu), Dholia-saturdoMoto-satoda (Gujarati), Snathikari (Hind), Kommegida (Canarese), Tambadivasu (Marathi) and Varshabhu (Sanskrit) (Singh, 2007).

Genus *Boerhaavia*, comprising of 40 species is distributed in humid and subtropical regions and warm climate. It is found in Ceylon, America, Malay Peninsula, Australia, Sudan China, Africa and Islands of the Pacific. 6 of the 40 species of *Boerhaavia* are found in India,

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namely *B. diffusa*, *Boerhaavia chinensis*, *Boerhaavia rubicunda*, *Boerhaavia hirsute*, *Boerhaavia erecta* and *Boerhaavia rependa*. Plant in India is found in hotter regions of the country and at the heights of 2,000 m in the Himalayan region. It is a persistent, dispersed hogweed, commonly occurring plentifully in waste places, waterways and dark places during rains (Najam et al., 2008).

### MORPHOLOGICAL PROFILE OF *B. DIFFUSA*

It is a diffusely branched, young and straight herb. It shows a mode of vertical growth and covered axis which grows loose and the renewable shoots rise on the upper surface of it (Anonymous, 1999; Chaudhary, 2010).

#### Roots

Roots are firm, tuberous, plump, rod-shaped, thick, fusiform with woody stock and bearing rootlets. They are light yellow or brownish gray in color having unpleasant taste and contain 0.15% alkaloid named *B. diffusa* (Bhansali et al., 1978; Shrivastava and Padhya, 1995). 2 to 3 layers of parenchymatous cells are present in cortex, followed by 5 to 12 layers of thin-walled, oval to polygonal cells, numerous concentric bands of xylem tissue alternating with extensive region of parenchymatous tissue present below cortical regions, vessels generally found in groups of 2 to 8 in radial rows, having simple pits and reticulate thickening, tracheids, small, thick walled with simple depths, fibre prolonged, thick-walled, spindle shaped with pointed ends, phloem occurs as curved patches outside each group of xylem vessels and composed of sieve elements and parenchyma, wide zone of parenchymatous tissue in between two repeated rings of xylem elements composed of thin-walled more or less rectangular cells arranged in radial rows, central regions of root occupied by primary vascular bundles, several raphides of calcium oxalate, in single or in group present in cortical region and parenchymatous tissue in between xylem tissue, starch grains simple and compound having 2 to 4 components found in abundance in most of cells of cortex, xylem elements in parenchymatous tissue between xylem elements, simple starch grains mostly rounded in shape and measure 2.75 to 11  $\mu$  in diameter.

#### Leaves and seeds

The leaves are ovate-oblong, fleshy, or subcordate at the base and smooth above, having flat margins. The upper surface of the leaves is green, smooth and glabrous, though it is hairy white and pinkish below. Spongy parenchyma 2 to 4 layered with small air spaces, idioblasts

comprising raphides, irregularly cluster crystal of calcium oxalate and orange-red resinous matter is present in mesophyll. Palisade ratio 3.5 to 6.5, stomatal index 11 to 16, and vein islet number 9 to 15. Cooked parts of plants are leaves, roots and seeds. They frequently can be ground into powder to complement with cereals when preparing bakery items (Anonymous, 1999). The seeds bud before the start of the rainy season.

#### Stem

Greenish purple, inflexible, trim, tube-shaped, swollen at nodes, young glabrous, often more than a meter long. Comprising of 9 to 12 stalked cells and an ellipsoidal head cortex which comprises 1 to 2 layers of parenchyma, pericycle 1 to 2 layered, thick-walled often comprising dispersed secluded fibers, stele containing countless small vascular bundles frequently linked together in a ring and several large vascular bundles spread in the ground tissue.

#### Flowers and fruits

Flowers are subcapitate, tiny, fascicled and pink. It is corymb, axillary and in terminal panicles, bracteoles, minor, acute, perianth tube limited above the ovary, inferior part greenish, ovoid, grooved, upper part pink, funnel-shaped, 3 mm long, tube 5 lobed, stamen 2 to 3. Fruit is glandular, narrowly oblong obovoid. Nut is one seeded, clavate is 6 mm in length, curved, broadly and bluntly 5 ribbed, viscidly glandular. These are sexless, pedicellate, pink or pinkish-red or white in color. In the place of a calyx and corolla; perianth is present, which is cylindrical in shape, the tube being short and narrow at the base and funnel-shaped at the top and surrounded above the ovary. The stigma is peltate. The achene fruit is removable, ovate, rectangular, young, five-ribbed and glandular, anthocarpous, and thick on the ribs (Thakur et al., 1989).

### PHYTOCHEMISTRY

Flavonoids, alkaloids, steroids, triterpenoids, lipids, lignins, carbohydrates, proteins, and glycoproteins are present in this plant in large quantity. Punarnavine, boeravinone A-F, hypoxanthine 9-larabinofuranoside, punarnavoside, lirodendrin, ursolic acid and a glycol-protein having a molecular weight of 16 to 20 kDa have been isolated and studied in detail for their biological activity (Ahmad and Hossain, 1968; Lami et al., 1990; Mishra and Tiwari, 1971; Aftab et al., 1996). The herb and roots are rich in proteins and fats. Many retinoids have been isolated from the roots of the *B. diffusa* (Ahmed

et al., 1990; Lami et al., 1990; Lami et al., 1991; Kadota et al., 2012). *B. diffusa* also contains many boeravinones that is, boeravinone A, boeravinone B, boeravinone C, boeravinone D, boeravinone E and boeravinone F (Seth et al., 1986; Jain and Khanna, 1989). C-methyl flavone also has been isolated from *B. diffusa* roots (Guptha and Ahmed, 1984). Two known lignans including, liriodendrin and syringaresinol mono-D-glycoside, purine nucleoside hypoxanthine 9-L-arabinose dihydroisofuroxanthone-borhavine; phytosterols about 0.04% of alkaloids known as punarnavine and punernavoside, about 6% of potassium nitrate, an oily substance and ursolic acid (Ojewole and Adesina, 1985; Lami et al., 1991; Ahmed and Yu, 1992). Phytochemical screening of the roots showed that the maximum alkaloidal contents (2%) gathered in the roots of 3-year old ripe plants. The herb comprises 15 amino acids, including 6 essential amino acids, while the root contains 14 amino acids.

## ETHNOMEDICINAL USES

The Unani and Ayurvedic preparations made from *B. diffusa* are used for the management of several illnesses that is, anemia, stomachache, cough, cold, an expectorant and laxative. Roots are used as diuretic, laxative, expectorant and the leaves are used as an appetizer and alexiteric preparation. Seeds are used as energizer and for help in digestion (Aslam, 1996). It was included in list of TRAMIL, a research project on the medicinal plant resources of the Caribbean (Robineau and Soejarto, 1996), because of its use in Martinican as folk medicine agent for the treatment of pain in general and of sore throat in the form of juice of fresh leaves (Robineau, 1995). In Brazil, the plant was in use in the traditional medicine as diuretic (roots) and against snake venom (Lorenzi, 1994). The leaves of *B. diffusa* are used as a green vegetable in many parts of India. It cures ulcers of cornea, night blindness and helps to bring back virility in men (Gupta et al., 1962). People in tribal areas use it to accelerate childbirth (Shah et al., 1983). The plant is also consumed as vegetable as it is thought to be a rich resource of vitamins, minerals, protein and carbohydrate (Cho et al., 2004).

## PHARMACOLOGICAL AND BIOLOGICAL ACTIVITY

The plant has gained lot of importance in the field of phytochemistry because of its various pharmacological and biological activities such as immunomodulatory effects, immunosuppressive, antimetastatic, antioxidant, antidiabetic, antiproliferative, antiestrogenic, analgesic, anti-inflammatory, antibacterial, antistress and adoptogenic activities. It is also reported to possess antilymphoproliferative, nitric oxide scavenging, hepatoprotective, anti-viral,

bronchial asthma, anti fibrinolytic activities, chemopreventive action, genetic diversity analysis and anticonvulsant activity.

### Antidiabetic activity

*B. diffusa* and ethanolic extracts exhibit significant antihyperglycemic activities in alloxan induced as well as streptozotocin induced hyperglycemic rats. They can also improve the condition of diabetes as indicated by parameters like body weight along with serum cholesterol and triglyceride levels. In the current studies, the damage of pancreas in streptozotocin treated diabetic control rats and regeneration of cells by glibenclamide was observed. The comparable regeneration was also shown by methanolic extracts of *B. diffusa* (Bhatia et al., 2001). A study was carried out to investigate the effects of daily oral administration of aqueous solution of *B. diffusa* L. leaf extract (BLEt) (200 mg/kg) for 4 weeks on blood glucose concentration and hepatic enzymes in normal and alloxan induced diabetic rats. A significant decrease in blood glucose and significant increase in plasma insulin levels were observed in normal and diabetic rats treated with BLEt (Pari and Satheesh, 2004). Chloroform extract of *B. diffusa* leaf produced dose-dependent reduction in blood glucose in streptozotocin-induced non-insulin-dependent diabetes mellitus (NIDDM) rats comparable to that of glibenclamide. The results specify that the reduction in blood glucose produced by the extract is possibly through renewal of pancreatic beta-cells or through extra pancreatic action (Nalamolu et al., 2004).

### Antibacterial activity

A strong antibacterial activity against gram positive and gram negative bacteria shown by the leaves of *B. diffusa* might be due to the phytochemicals present in the leaves. Ethanol extract exhibited inhibitory effect on gram positive bacteria like *Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Micrococcus luteus* and all gram-negative bacteria selected for the present study. Methanol extract showed inhibitory effect against all gram-positive bacteria selected for the present study except *M. luteus* and gram-negative bacteria like *Klebsiella pneumoniae*, *Proteus vulgaris*, *Serratia marcescens* and *Shigella flexneri* (Sharma et al., 2008). The aqueous and ethanolic extracts of *B. diffusa* leaves had action on *Escherichia coli*, *S. aureus* and *Pseudomonas aeruginosa*. This activity occurred at varying concentrations, indicating that the plant extracts contain active principle with wide antibacterial spectrum. *E. coli* displayed the highest susceptibility to ethanolic extract, followed by *S. aureus* and the least susceptible

was *P. aeruginosa*. In aqueous extract, *P. aeruginosa* showed the highest susceptibility, followed by *S. aureus* and *E. coli* exhibits the least receptiveness. The antimicrobial action of the different extracts increased with increase in concentration (Velmurugan et al., 2010; Sandhya et al., 2011).

#### **Antistress/ adoptogenic/ immunomodulatory activity**

Hydroethanolic extract (80%) of *B. diffusa* (HEBD) and a polyherbal formulation (Punarnava mandur) PHF-09 containing *B. diffusa* were compared for their antistress activity using cold restraint stress model. Stress was induced by subjecting animals to cold restraint. Due to cold restraint stress there was an imbalance in the levels of biochemical parameters like glucose, triglycerides, cholesterol, glutamic-oxalacetic transaminase (SGOT), glutamic-pyruvic transaminase (SGPT) activity which were near normalized following the administration of HEBD and PHF-09. HEBD and PHF-09 were found to have similar anti-stress activity (Akinnibosun et al., 2009). The ethanol extract of roots of *B. diffusa* was evaluated for antistress, adoptogenic activity in albino mice by swim durability test and cold restrains stress and the extract showed improved stress tolerance and immunomodulatory activity was observed in the form of increased carbon clearance, indicating stimulation of the reticuloendothelial system. There was a rise in DTH response to SRBC in mice, corresponding to cell mediated immunity and indicating stimulatory effects on lymphocytes and accessory cell types (Meera and Mustafa, 2007). *B. diffusa* (PUNARNAVA) has the ability to support both adrenal over and under activation. In stressful conditions, it has demonstrated the ability to safeguard against the elevations of serum cortisol and avoid the suppression of the immune system that takes place with raised cortisol. On the other hand, *B. diffusa* has also exhibited the ability to improve cortisol levels with end stage adrenal exhaustion (Mungantiwar et al., 1997).

#### **Hepatoprotective activity**

The hepatoprotective activity of roots of different diameters were collected in three seasons, rainy, summer and winter, and examined in thioacetamide intoxicated rats. The results showed that an aqueous extract (2 ml/kg) of roots of diameter 1 to 3 cm, collected in the month of May (summer), exhibited marked protection and majority of the altered serum parameters, that is, glutamic oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), acid phosphatase (ACP) and alkaline phosphatase (ALP), activity but not GLDH and bilirubin, thereby signifying the proper size and

time of collection of *B. diffusa* L. roots for the most wanted results. Further, the studies also proved that the aqueous form of drug (2 ml/kg) administration has more hepatoprotective activity than the powder form; this is perhaps due to the better absorption of the liquid form through the intestinal tract. The hepatoprotective activity of *B. diffusa* L. roots showed noticeable protection of serum parameters in thioacetamide toxicity in rats. Additionally, the aqueous extract of thin roots collected in the summer has more activity, suggesting the proper time and type of root collection for the most desirable result. The assessment also confirms the use of *B. diffusa* L. roots in hepatic illnesses by the several tribes in India (Rawat et al., 1997). An alcoholic extract of whole plant *B. diffusa* given orally exhibited hepatoprotective activity against experimentally induced carbon tetrachloride hepatotoxicity in rats and mice. The extract also produced an increase in normal bile flow in rats, signifying a strong choleric activity. The extract does not show any signs of toxicity up to an oral dose of 2 g/kg in mice (Chandan et al., 1991).

#### **Analgesic/anti-inflammatory activity**

The decoction (DE) or juice (JE) of the leaves of *B. diffusa* was used to study the antinociceptive effect in chemical (acetic acid) and thermal (hot plate) models of hyperalgesia in mice. The DE raised the pain thresholds during the first period (30 min) of observation. In the acetic acid-induced abdominal writhing in mice, pre-treatment of the animals with naloxone (5 g/kg, i.p.) significantly reversed the analgesic effect of morphine and JE but not that of DE. The study proves that the active antinociceptive principle of *B. diffusa* is present mainly in the juice of fresh leaves and has a significant antinociceptive effect when assessed in these pain models (Hiruma et al., 2000). Ethanol extract of leaves at dose of 400 mg/kg exhibited maximum anti-inflammatory effect with 30.4, 32.2, 33.9 and 32% with carrageenin, serotonin, histamine and dextran induced rat paw edema models, respectively. Ethanol extract of stem bark also showed COX-1 inhibitory activity with an IC<sub>50</sub> value of 100 ng/ml, proving the drug use in the treatment of inflammatory condition. Anti-inflammatory activity was measured using extract of latex of plant by using a carragenan induced inflammatory model (Krishna et al., 2010).

#### **Antitumor activity**

Cancer chemo preventive property of *B. diffusa* was assessed on 7,12-dimethyl benz (a) anthracene (DMBA) induced skin papillomagenesis in male Swiss albino mice (6 to 7 weeks old). The cancer chemopreventive efficacy

was assessed by its ability to lessen the activities of enzymes associated with drug metabolism and bifunctional modulators reduced the availability of final carcinogen metabolites in the epithelial stage. A significant increase in the activities of hepatic phase I, phase II system enzymes and antioxidant enzymes (glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase and glutathione level) were observed when mice were fed by oral gavage with *B. diffusa* extract at a dose level of 125 mg and 250 mg/kg body weight for a period of 14 days. It was inferred that the inhibition of tumorigenesis by the plant extract might have been due to preventing the formation of active carcinogens from their precursors or by augmenting detoxification process, preventing promotional events in the mouse skin through free radical scavenging mechanism (Rupjyoti et al., 2003).

#### Anti-convulsant activity

A study was carried out to investigate the methanolic root extract of *B. diffusa* and its different fractions including liriiodendrin-rich fraction for discovering the possible role of liriiodendrin in its anti-convulsant activity. Air-dried roots of *B. diffusa* were extracted with methanol by cold maceration. The methanol soluble fraction of extract thus obtained was continually extracted to obtain liriiodendrin rich fraction and two side fractions, that is, chloroform fraction and phenolic compound fraction. Anticonvulsant activity of methanolic extract and its different fractions, that is, liriiodendrin-rich fraction and phenolic compound fraction were studied in pentylenetetrazol (PTZ)-induced seizures. The crude methanolic extract of *B. diffusa* and only its liriiodendrin-rich fraction showed a dose-dependent protection against PTZ-induced convulsions. The liriiodendrin rich fraction showed significant protection against seizures induced by BAY k-8644. These findings reported the anticonvulsant activity of methanolic extract of *B. diffusa* roots and also it can be suggested that the observed anticonvulsant activity was due to its calcium channel antagonistic action as this activity was observed only in the liriiodendrin-rich fraction which has furthermore been established by significant anticonvulsant activity of liriiodendrin-rich fraction in BAY k-8644-induced seizures (Mandeep and Rajesh, 2011). Study showed that the crude methanolic extract of *B. diffusa* and its liriiodendrin-rich fraction showed a dose-dependent protection against PTZ-induced convulsions (Adesina, 1979).

#### Antiproliferative and antiestrogenic activity

This describes the antiproliferative and antiestrogenic properties of methanol extract of *B. diffusa* (BME) in MCF-7 breast cancer cell lines. *B. diffusa* extracts exhibited

a strong inhibitory effect on the production of human breast cancer cells *in vitro* and the antiestrogenic effects are mediated by ER. Phytochemical studies revealed the presence of alkaloids, flavonoids, phenols and saponins in BME. The antiestrogenic activity shown by the extract may be recognized to these diverse compounds (Sreeja and Sreeja, 1923).

#### Cytological activity

The extract of *B. diffusa* exhibited a strong depressive effect on the mitosis of *Crinum jagus* roots. The study was conducted using *B. diffusa* extract, the mitotic index of the control group was found to be 5.27. There was a negative association between the concentrations of the treatment extracts and the mitotic indices obtained from their action. This points to an inhibition of mitosis by this extract. Inhibition of the mitotic index increased significantly with an increase in the concentration of treatment solution of *B. diffusa*. Due to the ability of the root extracts of *B. diffusa* to collect metaphase and hence inhibit mitosis, it is possible to use these extracts as an alternative to the rather expensive colchicine for cytological studies (Ndubuisi et al., 2010).

#### Bronchial asthma

Dried leaves of *B. diffusa* can be used in dhoomapana in treatment of bronchial asthma. The leaf decoction is said to be an outstanding cough medicine when decocted with *B. diffusa* and then combined with ginger juice and black pepper (Sasi et al., 2009).

#### Anti fibrinolytic activity

A study evaluated the effect of antifibrinolytic agents;  $\gamma$ -aminocaproic acid ( $\gamma$ -ACA), tranexamic acid (AMCA); anti-inflammatory drugs (indomethacin, ibuprofen, naproxen) and plant extract (root extract of *B. diffusa*) on endometrial histology of inter uterine device (IUD)-fitted menstruating monkeys. It is effective in reducing stromal edema, inflammation, and tortuosity of glands, and in increasing the degree of deposition of fibrin and platelets in the vessel lumen (Barthwal and Srivastava, 1990).

#### Antioxidant activity

The assessment of the antioxidant potential of ethanolic extract of *Andrographis echioides* and *B. diffusa* was carried out by determining the levels of enzymatic and non-enzymatic antioxidants. The results showed that both the plant extracts possessed significant levels of enzymatic and non-enzymatic antioxidants. The results of the

**Table 1.** The composition of leaf of *B. diffusa*.

Leaf composition	Percentage
Moisture	82.20
Protein	06.10
Fats	00.90
Carbohydrates	07.20
Ash	00.96
Calcium	00.67
Phosphorous	00.99
Fiber	01.56

enzymatic and non-enzymatic antioxidants in *A. echinoides* and *B. diffusa* exhibits that they possess preventive and productive role to maintain the cell survival, cellular interaction and maintenance of cell membrane architecture. *A. echinoides* and *B. diffusa* have effective and therapeutic antioxidant potential against various inflammatory diseases (Premkumar et al., 2010).

The study was undertaken to evaluate antioxidant activity of chloroform, ethanol, and ethyl acetate fraction of *B. diffusa* L. roots which might have improved its hepatoprotective action. In *in vitro* nitric oxide scavenging activity, the percentage inhibition was 71.35, 33.74, 23.85% in ethanol, chloroform and ethyl acetate extracts at 250 µg/ml when compared with curcumin which at 62 mcg/ml showed 84.7% inhibition, respectively. The ethanol extract and ethyl acetate showed a biphasic response whereas the chloroform extract showed a dose dependent increase. In DPPH radical scavenging activity, the ethanol extract showed 81.94% inhibition and the chloroform extract showed 42.58% inhibition at 1000 mcg/ml compared with 88.02% inhibition by quercetin. The above results suggest that roots of *B. Diffusa* were found to show antioxidant potential which supports the use of this plant in traditional medicine (Gopal et al., 2010).

### Antiviral activity

Recently, strong antiviral effectiveness of this plant has been observed against phytopathogenic viruses. The antiviral agent isolated from this plant was found to be a glycoprotein with a molecular weight of 16 to 20 kDa. Administered by foliar spraying in the field, this antiviral agent could protect some economically important crops against natural infection by plant viruses. The aqueous extracts of plant material were prepared and tested *in vitro* as well as *in vivo* against phytopathogenic viruses on their hypersensitive and systemic hosts. The botanical identity of each plant was established before the extracts were prepared. Of the large number of plants screened, *B. diffusa* root extracts were found to have a broad spectrum

and very high antiviral activity (Awasthi and Verma, 2006).

### NUTRITIONAL VALUE

Different concentrations of vitamins are found in 100 g of dry plant that is, vitamin C = 44.80 mg, vitamin B3 = 97 mg and vitamin B2 = 22 mg. Mineral content per 100 g includes sodium 162.50 mg, magnesium 8.68 mg and iodine 0.002 mg (Kokate et al., 2005). 15 amino acids such as aspartic acid, proline, methionine, leucine, phenylalanine, glycine, serine, threonine, arginine, alanine, asparagine, valine, tyrosine and tryptophane are also present (Anonymous, 1999).

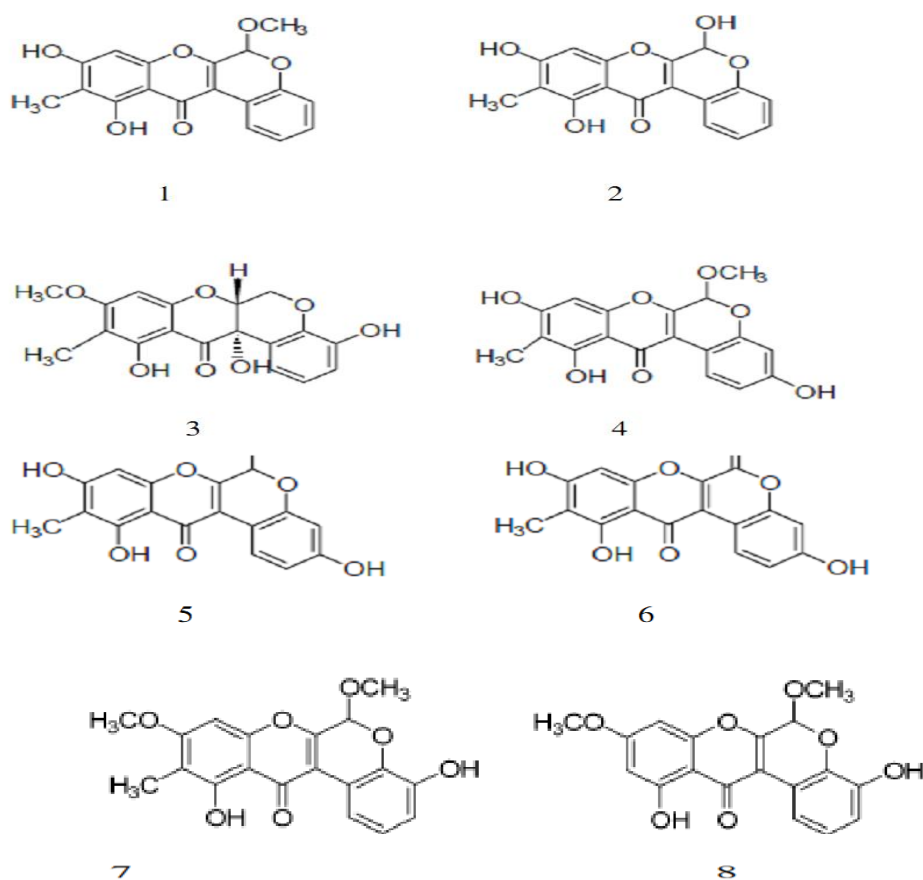
### COMPOSITION OF LEAF OF *B. DIFFUSA*

The composition of leaf of *B. diffusa* is shown in Table 1 (Ujowundu et al., 2008).

### CONSTITUENTS OF PHARMACOLOGICAL IMPORTANCE

Phytochemicals are natural bio-active compounds present in fruits, vegetables, plants and flowers. Leaves and fibers also play very important role in body defense system against diseases or more accurately protect plants against diseases (Krishnaiah et al., 2009). The therapeutic potentials including anti microbial, anti oxidant, anti cancer properties of higher plants are due to secondary metabolites (Caniguel et al., 2008; Kaur and Arora, 2009). Among very important bio active for example, flavonoids, alkaloids, glycosides and terpenoids. *B. diffusa* also contains a large number of flavonoids, retinoids, terpenoids, steroids, alkaloids (Kadota et al., 1989; Jain and Khana, 1989; Lami et al., 1990, 1991a). These biologically active compounds known as secondary metabolites in medicinal plants, form the foundations of modern prescription drugs (Sofwora, 1993).

It was discovered that multiple constituents are responsible for therapeutic effects of plant. These constituents act as a whole and it is very difficult to separate them as single. Moreover phytoconstituents in herbals mostly depend on plant origin, harvest season, time of cultivation, drying processes and other factors (Walker, 2004). First screening of *B. diffusa* revealed that it contains sterols (Singh and Udupa, 1972),  $\beta$ -sitosterol (Srivastava et al., 1972; Desai et al., 1973) and alkaloids. It contains about 0.04% of alkaloid known as punarnavine {C<sub>17</sub> H<sub>22</sub> N<sub>2</sub> O, mp (236 to 237°C)} (Surange and Pendse, 1972b) and punarnavoside, an anti fibrinolytic agent. Ursolic acid was also found in plant (Kokate et al.,



**Figure 1.** Main rotenoids (known as boeravinones) present in the root of *B. diffusa*. (1) Boeravinone A, (2) Boeravinone B, (3) Boeravinone C, (4) Boeravinone D, (5) Boeravinone E, (6) Boeravinone F, (7) Boeravinone G, (8) Boeravinone H. Source: Kadota et al. (1988a, b, 1989), Lami et al. (1990, 1991) and Borelli et al. (2005).

2005).

Research has demonstrated the presence of alkaloids and amino acids in *B. diffusa* (Garg, 1978, 1980). Two known lignans, liriodendrin and syringaresinol mono- $\beta$ -D-glucoside, have been isolated from a methanol extract of the roots of *B. diffusa* and the former compound was found to exhibit a significant calcium channel antagonistic effect (Lami et al., 1991). The seeds of this plant contain fatty acid and allantoin and the roots contain alkaloid (Aslam, 1996). The green stalk of the plant has also been reported to contain boehavin and boehavic acid (Liogier, 1990). Hentriacontane,  $\beta$ -sitosterol, ursolic acid along with some kinds of sugars were isolated from roots of *B. diffusa* (Misra and Tiwari, 1971). New C-methyl flavones characterized as 5, 7- dihydroxy-6-8-dimethoxy flavones have been reported from root (Gupta and Ahmed, 1984) and designated as boehavone (Ahmed and YU, 1992). Many rotenoid have been separated from roots of plant (Kadota et al., 1989; Lanni et al., 1990, 1991b). These include a series of boeravinone A, boeravinone B,

boeravinone C, boeravinone D, boeravinone E, boeravinone F, boeravinone G, boeravinone H (Figure 1).

Punarnavoside is present in roots and characterized as 2-glucopyrano-4- hydroxyl-5-(p-hydroxy phenyl) propionyl-diphenylmethane (Seth et al., 1986; Jain and Khana, 1989). Alcoholic extracts of *B. diffusa* leaves has immunosuppressive properties (Pandey et al., 2005). A new dihydroisofuraxanthone, methyl 3-10- dihydro-11-hydroxy- 1-methoxy-4-6- dimethyl- 10- oxo-1H-furo [3, 4-b] xanthenes-3-carboxylate, designated as boehavine has been extracted from benzene extract of roots of *B. diffusa* (Ahmed and YU, 1992). Various compounds like phenols, terpenes and volatile constituents were identified by using headspace–solid phase microextraction–gas chromatography–tandem mass spectrometry (HS-SPME-GS-MS). Moreover, numerous phenolic acids and flavonoids were also confirmed from study like quercetin and kaempferol (Pereira et al., 2009).

Two quinolized alkaloids were identified as Punarnavaine I and Punarnavaine II. Their distribution in

stems was maximum but minimum in roots. Content which is primarily low steadily increases during end of reproductive stage (Nandi and Chatterjee, 1974).

### TOXICITY OF *BOERHAVIA DIFFUSA*

Toxicological studies done on *B. diffusa* confirmed the absence of teratogenic and mutagenic effects and ingestion of bigger doses is linked with nausea (Singh, 1991; Patwardhan et al., 2004)

### EFFECT OF SEASONAL CHANGES AND ROOT THICKNESS ON HEPATOPROTECTIVE ACTIVITY OF PLANT EXTRACT

Hepatoprotective activity of plant is better in aqueous form (2 ml/kg) than powdered form which shows that the aqueous form of drug administration is better. Undoubtedly, it might be due to the improved absorption of the liquid form of the drug from the intestinal tract. On the other hand, high levels of serum marker hepatic enzymes were significantly reduced by the thin root extract (aqueous) of *B. diffusa* L. as compared to thick roots, signifying that the drug resultant from the thin roots has more hepatoprotective effect. Moreover, differences in hepatoprotectivity linked with adjustment in seasons. Protection of the common serum marker hepatic enzymes is greater in summers, as equated to the other, recommending it to be an appropriate time for gathering of the herb (Rawat et al., 1997).

### CONCLUSION

The plant in whole or its uncommon parts (aerial parts and roots) have plentiful medicinal properties and are used in widespread manner by ethnic people. The investigated and reported pharmacological and therapeutic properties of *B. diffusa* are; laxative, immunomodulatory activities, cancer chemo-preventive efficacy, anti-inflammatory, inhibition of tumor genesis, antioxidant activity, genoprotective, hepatoprotective activity, splenomegaly, hypoglycemic activity, antifungal, anti-proliferative, anti-estrogenic, morphinomimetic central analgesic property, potent anti-fibrinolytic, antibacterial activity, anticonvulsant activity, diuretic, spasmolytic activity, antinociceptive, anti-stress and dyspepsia. *Boerhaavia* species has been studied with deep interest due to its promising medicinal values in phytochemical and pharmacological research field. The objectives of review on *B. diffusa* is to sum up plant morphology, chemical compositions, its ethno-medicinal uses, linked from ancient times to the present, with a scope of development in future and this will be supportive to ascertain a standard

a standard likely drug for auxiliary exploration. The various benefits of *B. diffusa* made it a true wonder of nature.

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*Full Length Research Paper*

## Evaluation of spermatogenesis in prepubertal albino rats with date palm pollen supplement

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It is suggested that Date Palm Pollen (DPP) increases the concentration of testosterone, follicle stimulating hormone, and luteinizing hormone in rats. We therefore planned this study to examine the effect of DPP on spermatogenesis using prepubertal rats. 4-day old rats were randomly divided into four groups of 12 pups each (Control I, Experimental I, Control II, and Experimental II). Experimental groups I and II were given DPP suspension in a single oral dose of 120 mg/kg daily for 18 and 35 days, respectively. Pups were sacrificed on days 22 and 39 post-natally. Testes were removed for microscopic studies, and spermatogenesis was assessed by a method which depended upon scoring 'cross sectional' profiles of seminiferous tubules according to Johnsen's criterion. There was no statistically significant difference in Control and Experimental groups I. However Pups of the treated group II, who received DPP for 35 days, showed statistically significant increase in seminiferous tubules containing spermatozoa, resulting in a higher Johnsen Score (Control group II:  $6.28 \pm 0.21$ , Experimental group II:  $8.06 \pm 1.21$ ). This implied that spermatogenesis was present at an advanced stage in the Experimental group as compared to the Control group of animals.

**Key words:** Spermatogenesis, infertility, testes.

### INTRODUCTION

Throughout the world, 50 to 80 million couples suffer from infertility. Male factors are thought to be the major cause of infertility in 30% of cases and contribute to infertility in another 20% (Amelar et al., 1977). Infertility is commonly defined as the failure of conception after at least 12 months of unprotected intercourse (Pant, 2009).

Permanent infertility might be rare, but it is irrelevant to infertile couples who want a child, or to those who already have a child but are unable to conceive again (Hull et al., 1985). It is suggested that around 14 to 17% of couples may be affected at some time in their reproductive lives. Factors like diabetes, bronchiectasis,

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high grade fever, long term medication, urinary tract infection, sexually transmitted diseases, epididymitis, testicular injury, un-descended testis, mumps, orchitis, excessive alcohol, smoking, exposure to heat and certain chemicals affect spermatogenesis (Pant, 2009). The idiopathic causes of infertility are oligospermia, asthenozoospermia, teratozoospermia, azospermia etc. Others are obstructive azospermia, isolated seminal abnormalities, sexual or ejaculatory dysfunction and delayed puberty (Pant, 2009). Simply put, male infertility is a failure to fertilize the normal ovum due to a deficiency of functionally competent sperm at the stage of fertilization. The treatment modalities for male infertility include Intracytoplasmic sperm injection (ICSI) as an effective treatment modality for male factor infertility. However, this treatment could promote the transgenerational transmission of genetic defects causing gametogenic failure. The pharmacological treatments include clomiphene citrate, an anti-estrogen, the most commonly prescribed medication for male infertility, and L-carnitine known to improve semen's parameters, which has been recognized as the most effective medication (Meschede et al., 2000). There is not yet sufficient evidence for the routine use of other therapies (Ambler, 2009).

The beneficial health and nutrition values of *Phoenix dactylifera* L. for human and animal consumption have been claimed for centuries (Barrevel, 1993). Experimentally, date extracts have shown to increase sperm count in guinea pigs; and to enhance spermatogenesis and increase the concentration of testosterone, follicle stimulating hormone (FSH), and luteinizing hormone (LH) in rats (El-Mougy et al., 1991). The property of Date Palm pollen to increase serum testosterone level was evaluated in a parallel study (Iftikhar et al., 2011). The present study was designed to see the effect of DPP on the spermatogenesis of prepubertal rats with hopes to add a valuable contribution in advancement to the treatment of infertility.

## MATERIALS AND METHODS

This study was carried out at University of Health Sciences (UHS) Lahore, Pakistan.

### Preparation of the herbal cocktails

Date Palm pollen was obtained from Dera Ghazi Khan District of Punjab, through University of Health Science, Pakistan and was grounded into powdered form in the Pharmacology laboratory, UHS. It was mixed in distilled water to form a suspension. The dose was adjusted daily according to the body weight of the pups during the course of treatment and administered orally.

### Sample size

A total of 48 prepubertal male albino rats weighing 5 g were divided

into four groups, each group consisting of 12 rats. They were kept in the experimental research laboratory of UHS.

### Parameter studied

#### Johnsen's score

Johnsen's criterion was selected as it offers a convenient and rapid method for registration of spermatogenesis.

### Experimental procedure

Twelve female and four male adult albino rats were procured from National Institute of Health, Islamabad and were kept for two weeks in Experimental research laboratory of University of Health Sciences for acclimatization. One male and three female rats were housed together in a single cage for mating. Pregnancy was confirmed by observing vaginal plug in the morning after keeping the female and male rats together (Sato et al., 2005). Rats were monitored at 8 h interval to observe the time they deliver (Dhungel et al., 2006). Neonates were born after 21 days; they were kept with their mothers and examined for any congenital anomaly. Each of the 48 male neonates so obtained was given identification mark and divided randomly into following four groups of 12 pups each.

**Control I:** Received equal amount of distilled water daily as experimental animals for 18 days starting at 4th day of age.

**Control II:** Received equal amount of distilled water daily as experimental animals for 35 days starting at 4th day of age.

**Experimental I:** Received 120 mg/kg body weight of DPP suspension (Bahmanpour et al., 2006a), as a single oral dose daily for 18 days starting at 4th day of age (Pico et al., 2007).

**Experimental II:** Received 120mg/kg body weight of DPP suspension (Bahmanpour et al., 2006b), as a single oral dose daily for 35 days starting at 4th day of age (Pico et al., 2007).

Each group was kept under controlled room temperature ( $22 \pm 2^\circ\text{C}$ ) and humidity of  $55 \pm 10\%$ . They were kept under natural light and dark cycle. All pups were fed on mother's milk and gradually weaned to normal rat chow and water *ad libitum*. The mothers were, however, fed *ad libitum* on normal rat chow and water. 4-day old rats were selected since the experiment consumed 35 days in total. The pups of Control I and Experimental I groups were weighed and sacrificed on 22nd day post-natally, after 18 days of treatment, to determine if DPP affected the mentioned parameters in the middle of the experimental period, and those of Control II and Experimental II were sacrificed on 39th day post-natally, after 35 days of treatment (Bahmanpour et al., 2006b).

### Dissection

Each animal was anesthetized with chloroform. A vertical midline skin incision was given from xyphoid to symphysis pubis; it was extended laterally by a transverse incision on each side of the midline. The skin was reflected laterally and abdomen was opened; both skin and muscles were removed. The testes were retractable and were pushed forward into the body cavity. They were removed by pulling the tails of the epididymides along with their head and body, vas deferens and spermatic blood vessels. The blood vessels and vas deferens were severed allowing removal of the testes and the epididymides (Crawford, 2008).

**Table 1.** Scoring 'cross-sectional' profiles of seminiferous tubules.

s/No.	Parameter
10	Complete Spermatogenesis with many spermatozoa
9	Many spermatozoa present but disorganized spermatogenesis
8	Only a few (<5) spermatozoa
7	No spermatozoa but many spermatids
6	No spermatozoa and only a few (<5) spermatids
5	No spermatozoa or spermatids but several or many spermatocytes
4	No spermatozoa or spermatids and only a few (<5) spermatocytes
3	Spermatogonia are the only germ cells present
2	No germ cells but sertoli cells are present
1	No cells in tubular section

### Histological techniques

The right testis of each animal was sectioned along the midline (Rhoden et al., 2002) and immersed in Bouin's fixative for 24 h (Çiftçi, 2004; Udagawa et al., 2006). Testis was then washed with changes of 50 and 70% ethanol for 72 h to remove the yellow color. After washing, each half of the testis was placed separately in a single tissue cassette. The tissue was processed in the automatic tissue processor (UTECH Product INC. Albany, New York-12203 USA) for 18 h, dehydrated by passing through ascending grades of alcohol using 50, 70, 90% and absolute ethanol, cleared in xylene and infiltrated with molten paraffin wax (58°C) before preparing the block (Bancroft and Gamble, 2008). Paraffin block was prepared by placing the tissue in a base mold. The block was firmly fixed in the chuck of the Leica RM 2125 rotary microtome (Leica Biosystems). Five micrometer thick sections were obtained; these were shifted to water bath kept at 45 to 50°C, folds were removed using camel hair brush. Sections were then transferred on the surface of clean and albumenized glass slides, labeled with diamond pencil. Excess of water was drained off and were then dried on the slide warmer.

### Scoring of the tubules

The H&E stained slides were observed under light microscope (OLYMPUS CX31, Model U-DO3, OLYMPUS Japan) at different magnifications. The histological findings were recorded and interpreted according to the method reported by Johnsen (1970). This envisaged scoring 'cross-sectional' profiles of seminiferous tubules according to the criteria given in Table 1. The scoring was performed at x40 objective exposing several tubules in one field. In case of doubt, the presence of spermatozoa was checked by higher magnification. Tubules in one field, chosen at random, were scored and the slide was then moved sideward to bring the adjacent area within the field. When coming to the edge of the section, the slide was moved up to bring the structure at the top edge to the bottom edge and scoring continued. Damaged tubules, at the edges of the section were rejected.

Ten seminiferous tubules in each section were scored. One stained slide from each of the forty eight animals was examined and four hundred and eighty observations were made. In order to calculate a mean score, the number of tubules recorded at each Johnsen score was multiplied with the score and the sum of all 10 multiplications was divided by the total number of tubules recorded (Johnsen, 1970; Carroll et al., 1997).

### Statistical analysis

The statistical analysis was carried out using computer software 'Statistical Package for Social Sciences (SPSS) version 18.0'. The arithmetic mean of observations and standard deviation values were calculated; two independent samples t-tests (null hypothesis) were applied to observe differences in mean values. The difference was regarded statistically significant if the 'p' value was < 0.05.

### Ethical considerations

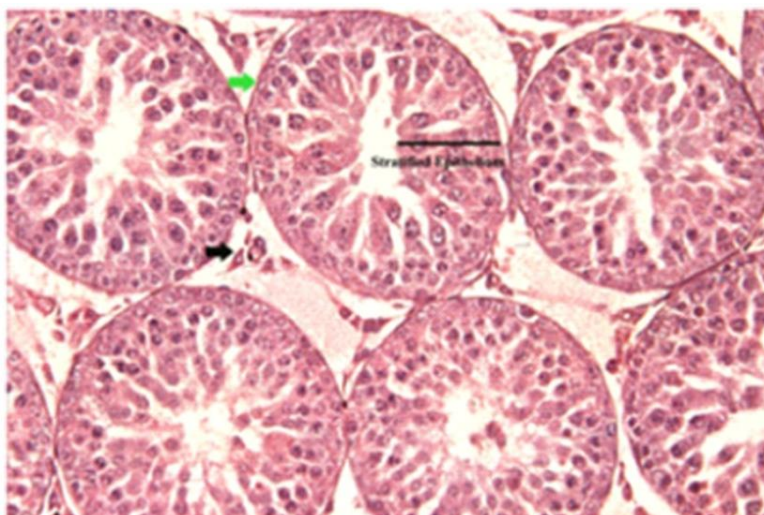
These were according to the instructions given in World Health Organization Declaration of Helsinki.

1. During the experiment, the health of the animal will be of paramount importance. Animals showing any sign of disease will be removed from the experiment
2. Proper diet will be maintained during the experiment.
3. Animals will be sacrificed with utmost regard to keep the process painless. Proper disposal of the dissected animal will be done.

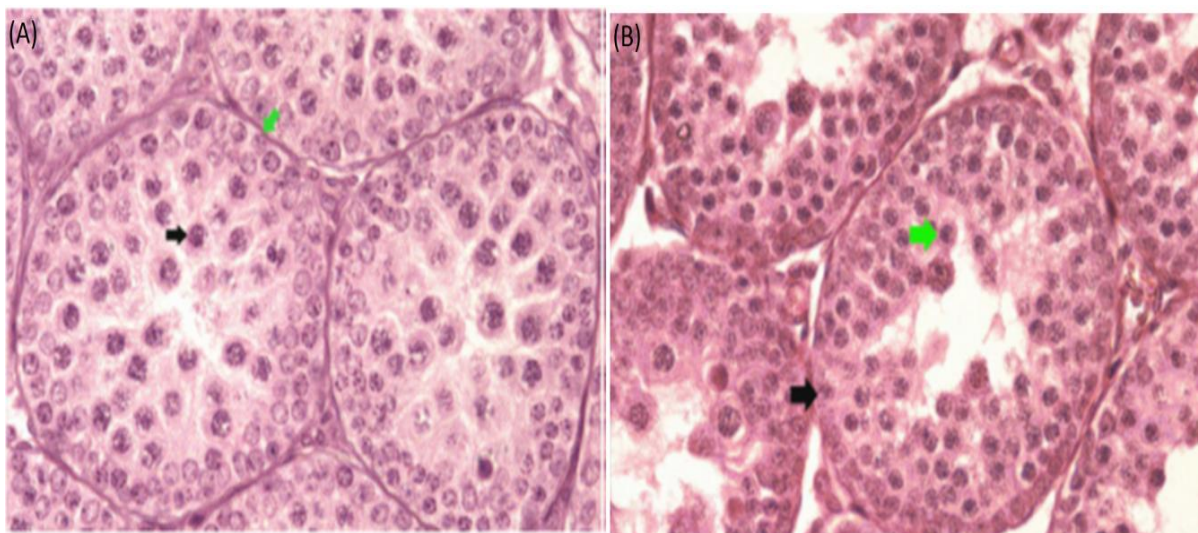
## RESULTS

### Histological observations of testes

The gland was covered by connective tissue capsule which contained fine reticular connective tissue stroma. Each testis was divided into lobules by incomplete connective tissue septa that projected from the capsule. Seminiferous tubules were lined with stratified epithelium in all the groups; connective tissue stroma formed their interstices. Leydig cells, spherical or ovoid in shape, with a central darkly stained nucleus and lighter staining vacuolated cytoplasm were seen within the inter-tubular tissue of Control and Experimental groups. Sertoli cells with oval euchromatic nuclei containing prominent nucleoli and some apparent eosinophilic stained cytoplasm could be seen resting on the basement membrane (Figure 1).



**Figure 1.** Photomicrograph of a prepubertal testis showing normal architecture. Seminiferous tubules are surrounded by regular and thin basement membrane (green arrow). Leydig cells (black arrow) are arranged in groups. Tubules are lined with stratified epithelium. H&E stain. X200.

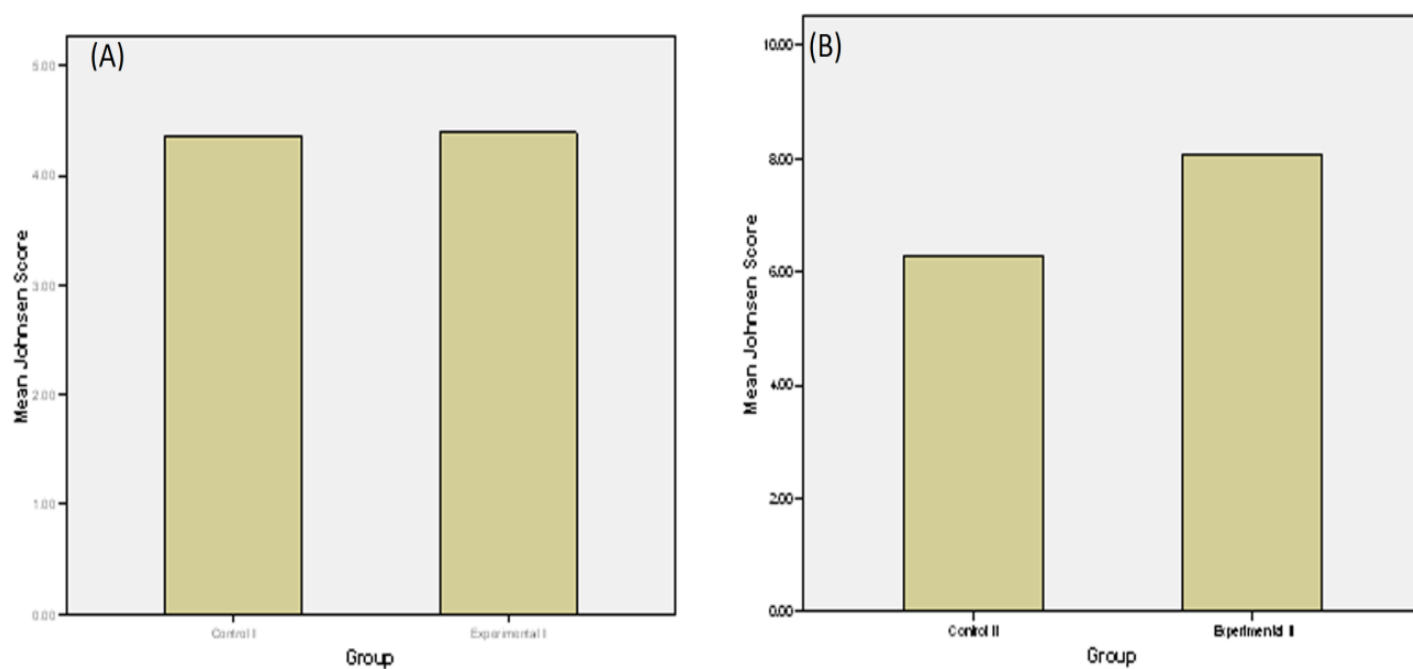


**Figure 2.** (A) Photomicrograph of testis from Control group I illustrating seminiferous tubule at Johnsen Score 5 which contains spermatogonia (green arrow) and spermatocytes (black arrow) H&E stain. X400. (B) Photomicrograph of testis from Experimental group I illustrating seminiferous tubule at Johnsen Score 5 which contains spermatogonia (black arrow) and spermatocytes (green arrow) H&E stain. X400.

### Johnsen score (18 days therapy)

In the Control and Experimental groups I, the most advanced cells seen were spermatocytes, some were in the process of division. Primary spermatocytes, the largest cells of spermatogenic lineage, characterized by the presence of chromosomes in various stages of the coiling process within their nuclei were identified in both

the groups; their nuclei were large with poorly packed chromatin (Figure 2A and B). In the animals of Control group I, Johnsen score of testes varied from 4.1 to 4.6, with an average of  $4.37 \pm 0.14$ ; in the treated group I it ranged from 4.2 to 4.6 with the average of  $4.39 \pm 0.15$ . Difference in Johnsen scores of animals of Control and Experimental groups I was not statistically significant  $p = 0.681$  (Figure 3A and Table 2). Spermatogonia, roughly



**Figure 3.** (A) Mean Johnsen scores of animals of control I and experimental I Groups after 18 days therapy. (B) Mean Johnsen Scores of animals of control II and Experimental II groups after 35 days therapy.

**Table 2.** Comparison of Mean Johnsen scores of animals among experimental and control Groups I after 18 days Therapy.

Parameter	Control Group I (12) Mean $\pm$ SD	Experimental Group I (12) Mean $\pm$ SD	p-value
Johnsen Score	4.37 $\pm$ 0.14	4.39 $\pm$ 0.15	0.681

Figure in parenthesis indicate total number of animals in each group.

**Table 3.** Comparison of mean Johnsen scores of animals among experimental and control Groups II after 35 days therapy.

Parameter	Control Group II (12) Mean $\pm$ SD	Experimental Group II (12) Mean $\pm$ SD	p-value
Johnsen Score	6.28 $\pm$ 0.21	8.06 $\pm$ 1.21	<0.001

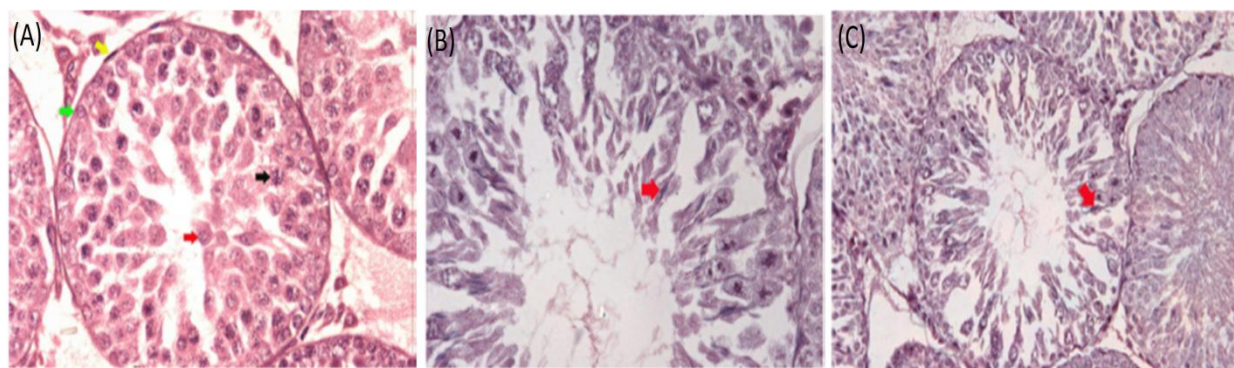
Figure in parenthesis indicate total number of animals in each group.

spherical in shape, with normal nuclear and cytological morphologies were seen next to the basement membrane; they contained finely granular chromatin within a small nucleus in all the groups.

### Johnsen score (35 days therapy)

In the animals of Control group II, Johnsen Score of testes varied from 6.0 to 6.5 with an average of 6.28  $\pm$  0.21; in the treated group II it ranged from 6.4 to 9.2 with

the average of 8.06  $\pm$  1.21. Difference in Johnsen scores of animals of Control and Experimental groups II was statistically significant  $p < 0.001$  (Figure 3B and Table 3). The cells of spermatogenic lineage-spermatogonia, spermatocytes and rounded spermatids were stacked in several layers in seminiferous tubules of Control and Experimental groups II. In the Control and Experimental groups II, close to the lumen were the small, pale staining nuclei; these belonged to spermatids. However, seminiferous tubules of Experimental group II showed presence of spermatozoa within the lumen, implying thereby that



**Figure 4.** (A) Photomicrograph of testis from Control group II illustrating seminiferous tubule at Johnsen Score 7 which contains spermatogonia (green arrow), spermatocytes (black arrow) and spermatids (red arrow). Myoid cell (yellow arrow) is visible in the peritubular area. H&E stain. X400. (B) Photomicrograph of testis from Experimental group II illustrating seminiferous tubule at Johnsen Score 10 containing spermatozoa (red arrow). H&E stain. X400. (C) Photomicrograph of testis from Experimental group II illustrating seminiferous tubule containing spermatozoa (red arrow) at a lower magnification. H&E stain X200.

spermatogenesis was present at an advanced stage in the Experimental as compared to the Control group II of animals. The cytoplasmic and nuclear structures of all the cells were normal in both groups (Figure 4A to C).

## DISCUSSION

Date palm pollen had a positive effect on spermatogenesis in the rat model used. Infertility related stress is considered second to that involving the death of a family member or divorce by couples. It is ranked as one of the greatest source of stress in a person's life (Anderheim et al., 2005, Roudsari et al., 2007). Experimentally, date extracts have been shown to increase the concentration of testosterone, follicle stimulating hormone, and luteinizing hormone in rats (El-Mougy et al., 1991). However, there is not much literature or data available regarding its effect on spermatogenesis of prepubertal animals; therefore, we conducted this study on prepubertal rats to assess its effect.

In this study, spermatogenesis was assessed to see the effect of DPP suspension (120 mg/kg) given orally for 18 and 35 days in premature albino rats. Our data showed that there was no statistically significant difference in the observed parameter after 18 days of treatment with Date Palm pollen suspension in Control and Experimental groups I. It is suggested that processes controlling the absorption, distribution, metabolism, excretion, and pharmacologic effects of drugs are likely to be immature or altered in neonates (Milsap and Jusko, 1994). Moreover, the drugs administered to neonatal animals may exhibit significantly different pharmacokinetic/disposition characteristics than they do in adult animals of the same species (Schwark, 1992). Therefore, inability of DPP to produce its effect in the group treated for 18 days

may be due to age-related differences in maturation pathways responsible for drug absorption, distribution, metabolism and excretion (Espandari et al., 2010) or treatment with DPP took somewhere between 18 and 35 days to produce its effects.

Our data agrees with a study carried out by Bahmanpour Bahmanpour et al. (2006b), where they observed the effect of *Phoenix dactylifera* pollen on sperm parameters and reproductive system of adult male rats, after 35 days of treatment. There was a statistically significant increase in Johnsen's score in the group treated for 35 days with DPP suspension. Johnsen's criterion offers a convenient and rapid method for registration of spermatogenesis (Johnsen, 1970). Mean Johnsen score of control group II was  $6.28 \pm 0.21$ , whereas in the treated group II, it was  $8.06 \pm 1.21$ . The increase in the Johnsen's score of the treated group when compared with the Control group was statistically significant ( $p < 0.001$ ). Johnsen showed a high correlation between log total sperm count and the mean Johnsen score. Thus an increase in the mean Johnsen score in our study is an indirect evidence of improvement in the sperm count. Bahmanpour et al. (2006b) also reported that DPP suspension reduced the sperm DNA denaturation and therefore, seems to improve its DNA quality and sperm parameters.

These findings also agree with the findings of Arsyad (1996) who showed that protodioscin treatment led to increase in concentration of spermatozoa; there was also an increase in the mobility and percentage of normal sperm. The authors attributed this to an increase in the LH level which was responsible for the Leydig cells to enhance testosterone secretion, resulting in an increased testosterone level in the blood stimulating sertoli and germinal cells and as stated earlier, date palm extracts increase these hormones in rats (El-Mougy et al., 1991). The most advanced cell present in the seminiferous

tubules of Control group II were spermatids, whereas testes of Experimental group II showed presence of spermatozoa within the lumen, implying thereby that spermatogenesis was present at an advanced stage in the Experimental as compared to the Control group II of animals.

## Conclusion

Date Palm pollen suspension given orally at a dose of 120 mg/kg for 35 days resulted in an increase Johnsen scores. Further, spermatozoa were seen in the seminiferous tubules of Experimental group II. Our data regarding the effects of Date Palm pollen on testis implied that it had a complex stimulating effect on germinative and endocrine functions of the organ.

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