BIOLOGICAL AND PHYTOCHEMICAL STUDIES ON BOERHAAVIA DIFFUSA

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XX CICLO 2004-2007
ACKNOWLEDGEMENTS

The present thesis of PhD was carried out at the Department of Experimental Pharmacology, University of Naples Federico II, during the years 2004–2007.

I would like to express my warmest thanks and acknowledgements to all those who encouraged me to complete my thesis.

First of all I would like to extend my heartfelt thanks to Professor Francesco Capasso, for his supervision during the research and for motivating and inspiring me over the years to broaden my interests in plant drugs. His ideas made it possible to realise this thesis.

I owe my sincere thanks to Prof. Nicola Mascolo and Prof. Angelo Izzo who encouraged and advised me throughout the research work.

I would particularly like to thank Dr. Francesca Borrelli for her valuable guidance and crucial and constructive suggestions that greatly improved this study.

I would like to thank Dr. Raffaele Capasso and Dr. Gabriella Aviello for their considerate support, useful comments and friendship during the current research.

I owe my special thanks to Prof. Ernesto Fattorusso and Prof. Orazio Taglialatela-Scafati, Department of Chemistry of Natural Products, Faculty of Pharmacy, University of Naples Federico II for generous help and cooperation (phytochemical part) during the research work.

I would like to express my gratitude to Prof. Jasna Canadanovic-Brunet, Department of Organic Chemistry, Faculty of Technology, University of Novi Sad, Serbia, who gave me the opportunity to do some research experiments in the laboratory.

I would like to thank the coordinators of the doctorate study, Prof. Enrico Abignente and Prof. Maria Grazia D’Auria who took care of all the students problems with patience and generosity.

Last but not least, I sincerely thank all my colleagues and the staff members of the Department of the Experimental Pharmacology for the assistance, patience and understanding. They were always extremely helpful with this project.

Finally, I owe my loving thanks to my family for encouraging me in the most important decisions in my life.
CONTENTS

Summary in Italian I
Summary in English IV
Abbreviation VII

1.0 INTRODUCTION 1
  1.1 Ayurvedic Medicine 1
  1.2 Boerhaavia diffusa 5
    1.2.1 Botany 7
    1.2.2 Ethnobotanical Use 8
    1.2.3 Biological Activity 11
      1.2.3.1 Traditional Medical System 11
      1.2.3.2 Pharmacological and Clinical Properties 13

2.0 RESEARCH OBJECTIVES 19

3.0 MATERIALS AND METHODS 21
  3.1 Plant Material 21
  3.2 Studies on Intestinal Motility 22
    3.2.1 Substances and Drugs 22
    3.2.2 Bioassay-Guided Isolation of the Active Compounds 23
    3.2.3 Animals 29
    3.2.4 Guinea Pig Ileum Isolation 30
    3.2.5 Contractions Induction 30
  3.3 Antioxidant Activity 34
    3.3.1 Substances and Drugs 34
    3.3.2 Bioassay-Guided Isolation of the Active Compounds 34
    3.3.3 Cell Culture 36
    3.3.4 Electronic Spin Resonance Spectrometer (ESR) 37
    3.3.5 Sucrase - Isomaltase Activity Determination 40
    3.3.6 TBARS Assay 42
    3.3.7 Intracellular ROS Measurement 43
  3.4 Genoprotective Assay 45
3.4.1 Substances and Drugs 45
3.4.2 Bioassay-Guided Isolation of the Active Compounds 45
3.4.3 Comet Assay 45
3.5 BCRP Mediated Inhibition Assay 47
  3.5.1 Substances and Drugs 47
  3.5.2 Bioassay-Guided Isolation of the Active Compounds 47
  3.5.3 Cell Culture 51
  3.5.4 BCPR Inhibition 51
3.6 Toxicological Studies 52
  3.6.1 Substances and Drugs 53
  3.6.2 Bioassay-Guided Isolation of the Active Compounds 53
  3.6.3 MTT Assay 53
  3.6.4 Lactate Dehydrogenase (LDH) Leakage Assay 54
  3.6.5 Genotoxicity Assay (Comet Assay) 55
3.7 Statistical Analysis 56
4.0 RESULTS 58
  4.1 Spasmolytic Activity 58
  4.2 Antioxidant Activity 66
    4.2.1 Electron Spin Resonance Spectroscopy (ESR) 66
    4.2.2 Sucrase- Isomaltase Activity 73
    4.2.3 TBARS Assay 73
    4.2.4 Intracellular ROS Measurement 75
  4.3 Genoprotective Activity (Comet Assay) 78
  4.4 BCRP Mediated Inhibition Assay 83
  4.5 Toxicological Studies 85
    4.5.1 Cytotoxicity Assay 85
      4.5.1.1 MTT Assay 85
      4.4.1.2 Lactate Dehydrogenase (LDH) Leakage Assay 89
    4.4.2 Genotoxicity Assay (Comet Assay) 89
5.0 DISCUSSION 93
6.0 CONCLUDING REMARKS 115
7.0 LITERATURE 118
APPENDIX 133
Summary

La *Boerhaavia diffusa* (famiglia delle *Nyctaginaceae*) è una pianta erbacea perenne, nativa dell’India e del Brasile, dove è stata usata, per secoli, come pianta medicamentosa dalle popolazioni indigene. Le radici di *B. diffusa* dal punto di vista chimico contengono alcaloidi (punarnavine), rotenoidi (boeravinoni A-F), flavonoidi, amminoacidi, lignani (liriodendrine) β-sitosteroli ed acidi tetracosanoici, esacosanoici, stearici ed ursolici. Le radici di *B. diffusa* vengono utilizzate per il trattamento di numerose patologie, quali disordini epatici (ittero, epatite, ecc.), gastro-intestinali (come lassativo), disturbi renali (calcoli, cistiti e nefriti), nel trattamento dell’anemia e nella sindrome mestruale. Inoltre recentemente, tale droga è anche utilizzata come coadiuvante della terapia antitumorale. Nonostante il largo uso in terapia della *B. diffusa*, allo stato attuale non sono presenti in letteratura dati scientifici chiari che (i) dimostrino l’esistenza di un’attività farmacologica e (ii) spieghino il meccanismo d’azione. Sulla base dell’utilizzo della *B. diffusa* nella medicina Ayurvedica e della mancanza di dati scientifici che spieghino tale utilizzo, lo scopo del nostro studio è stato quello di: (i) valutare l’effetto di un estratto metanolico di *B. diffusa* sulla muscolatura liscia intestinale; (ii) studiare la potenziale attività antiossidante di tale estratto, (iii) individuare i metaboliti responsabili di tali attività farmacologiche, (iv) investigare l’azione di alcuni metaboliti attivi presenti nelle radici di *B. diffusa* (i boeravinoni) sull’attività di una classe di proteine note come proteine resistenti al cancro della mammella (Breast cancer resistance protein, BCRP), stabilendone una potenziale relazione struttura-attività, ed infine (v) valutare la potenziale attività citotossica dell’estratto in studio e dei metaboliti responsabili delle attività biologiche. **Materiali e metodi:** Studi sulla motilità intestinale. Sono stati condotti studi
in vitro sull’ileo isolato di cavia sottoposto all’azione contratturante di agenti esogeni (quali acetilcolina, istamina, BaCl₂) e della stimolazione elettrica (EFS). Attività antiossidante. Sono stati effettuati studi di Risonanza di Spin elettronica (ESR) e determinati i livelli di MDA e ROS in una linea cellulare di adenocarcinoma umano (Caco-2) sottoposta a stress ossidativo; è stata inoltre valutata la potenziale azione genoprotettiva dell’estratto attraverso il saggio della cometa (Comet assay). Effetto sulle BCRP. Su una linea embrionale umana di rene (HEK-293) è stato valutato l’effetto dei metaboliti attivi dell’estratto metanoloico delle radici di B. diffusa sull’accumulo del mitoxantrone. Studi di citotossicità. Sulle Caco-2 è stato effettuato il test dell’MTT e dell’LDH. Risultati: L’estratto di B. diffusa (1-1000 µg/ml) inibiva significativamente ed in maniera concentrazione dipendente le contrazioni indotte dagli agonisti esogeni e dalla stimolazione elettrica. Tale effetto inibitorio era ridotto in presenza di nifedipina ed EDTA, suggerendo un coinvolgimento dei canali del calcio di tipo L nel meccanismo d’azione della B. diffusa. L’estratto metanoloico di B. diffusa (0.1-5 mg/ml) riduceva significativamente ed in maniera concentrazione dipendente la formazione di radicali liberi (saggio dell’ESR); inoltre, l’estratto in esame così come le suesuccessive frazioni (30-300 µg/ml) riduceva (i) i livelli di malonildialdeide e di ROS indotti nelle Caco-2 dal reattivo di Fenton ed (ii) il danno al DNA indotto dall’H₂O₂. I boeravinoni isolati dall’estratto metanoloico di B. diffusa inibivano ed in maniera concentrazione dipendente l’escrezione del mitoxantrone mediata dalle proteine BCRP (i boeravinoni G ed H sono risultati più attivi). Infine, l’estratto di B. diffusa (30-300 µg/ml), non induceva effetti citotossici. Attraverso frazionamento guidato dai saggi biologici sono stati isolati 15 boeravinoni (dieci noti e 5 nuovi da noi denominati boeravinone G, boeravinone H, boeravinone I, boeravinone J e
6,10,11-tridrossi-9-metossiroteneone). **Conclusioni:** Nel presente studio è stato evidenziato che un estratto metanolico di B. diffusa (i) possiede attività spasmolitica sulla muscolatura liscia intestinale. Tale effetto, che potrebbe in parte giustificare l’uso tradizionale di tale pianta nel trattamento dei disturbi gastrointestinali, sembra dovuto alla presenza di boeravinoni e sembra coinvolgere i canali del calcio di tipo L; (ii) presenta un’azione antiossidante e genoprotettiva che potrebbe giustificare il suo impiego nei disturbi e nelle patologie legate a danni ossidativi. I boeravinoni (G ed H in particolare) sono in grado di contrastare il fenomeno di farmacoresistenza indotto da antitumorali.
Summary

*Boerhaavia diffusa* (Nyctaginaceae family) is a herbaceous perennial plant, native of India and Brazil, where it was used for centuries as a medicinal plant by indigenous populations. The root of *B. diffusa* contains alkaloids (punarnavine), rotenoids (boeravinones A-F), flavonoids, amino acids, lignans (liriodendrons), ß-sitosterols and tetracosanoic, esacosanoic, stearic and ursolic acids. The root of *B. diffusa* is used for the treatment of many diseases, such as liver disorders (jaundice, hepatitis, etc.), gastro-intestinal disorders (as laxative), renal disorders (for calculations, cystitis and nephritis), and for the treatment of anaemia and of menstrual syndrome. The drug has recently been used as an adjuvant in an anticancer therapy. Despite the wide therapeutical use of *B. diffusa*, there are still no scientific data in the literature which clearly (i) demonstrate the existence of a pharmacological activity and (ii) explain the mechanism of its action. Since *B. diffusa* is largerly used in Ayurvedic medicine and the scientific data that explain such use are deficient, the purpose of our study was: (i) to evaluate the effect of the methanolic root extract of *B. diffusa* (BDRME) on the intestinal smooth muscles, (ii) to study the potential antioxidant activity of the extract/fractions, (iii) to identify the metabolites responsible for such pharmacological activities, (iv) to investigate the action of some active metabolites present in BDRME (boeravinones) on the activity of the protein type known as the breast cancer resistance protein (BCRP) setting a potential relationship structure-activity, and finally, (v) to assess the potential cytotoxic activity of the studied extract and its metabolites responsible for the given biological activity. **Materials and Methods:** Studies on Intestinal Motility. The *in vitro* studies were conducted on the isolated ileums of guinea pigs subjected to the contractions by exogenous agents (such as ACh, histamine, BaCl₂) and the electrical stimulation (EFS).
Antioxidant Activity. The Electronic Spin Resonance (ESR) studies were conducted to determine the antiradical activity of the BDRME and of its fractions. The levels of MDA and ROS were determined in a human adenocarcinoma cell line (Caco-2) subjected to the oxidative stress; the potential genoprotective action of the extracts was also evaluated by Comet assay. Effect on BCRP. The effect of the active metabolites with mitoxantrone on the human embryonic kidney line (HEK-293) was assessed. Studies of Cytotoxicity. The MTT and LDH tests were conducted on the Caco-2 cells. Results: The BDRME (1-1000 µg/ml) significantly and in a concentration dependent manner reduced the contractions of isolated guinea pig ileum induced by the exogenous agonists and by EFS. This inhibitory effect was reduced in the presence of nifedipine and EDTA, suggesting the involvement of the calcium channels of the L-type mechanism of B. diffusa action. The BDRME (0.1-5 ml/ml) significantly reduced and in a concentration-dependent manner the formation of free radicals (ESR assay); moreover, the examined extract as well as its fractions (30-300 µg/ml) (i) reduced the levels of malonaldehyde and ROS induced in Caco-2 by Fenton reaction and (ii) the DNA damage induced by H₂O₂. The isolated boeravinones from the BDRME inhibited and in concentration dependent manner the excretion of mitoxantrone mediated by BCRP protein (boeravinones G and H were the most active). Finally, the BDRME (30-300 µg/ml) did not induce any cytotoxic effect. Through a bio-assay-guided separation, fifteen boeravinones were isolated (ten known and five new which we named boeravinone G, boeravinone H, boeravinone I, boeravinone J and 6-O-dimethylboeravinone H). Conclusion: In the present study it has been shown that BDRME (i) possesses spasmolitic activities on intestinal smooth muscles. This effect, which could partially justify the traditional use of this plant for the treatment of gastrointestinal disorders, seems to be due to the presence of boeravinones and their the involvement of extracellular calcium
and/or L-type calcium channels and (ii) possesses the antioxidant and genoprotective actions that could justify its use in diseases and pathologies induces by oxidative damage. The boeravinones (G and H in particular) are interesting BCPR inhibitors able to counter the phenomenon of drug resistance induced by anticancer drug.
ABBREVIATION

AA - Antiradical Activity
ABC - “ATP-Binding Cassette”
ACh - Acetylcholine
ATCC - American Type Cultures Collection
BOE - *Boerhaavia diffusa* sub-fraction
BSPR - Breast Cancer Resistant Proteins
Caco-2 - Human Colon Adenocarcinoma Cells
DCF - 2′-7′-dichlorofluorescin
DMPO - 5,5-dimethyl-1-pyrroline N-oxide
DMSO - Dimethyl Sulfoxide
DMEM - Dulbecco's Modified Eagle Medium
DNA - Deoxyribonucleic Acid
EDTA - Ethylenediaminetetraacetic Acid
EGTA - Ethylene glycol-bis (β-aminoethyl ether)-N,N,N’,N’-tetraacetic Acid
EIMS - Electron Ionization Mass Spectrometry
EFS - Electrical Field Stimulation
ESR - Electronic Spin Resonance
FBS - Foetal Bovine Serum
HEK-293 - Human Embryonic Kidney - 293
HPLC - High Pressure Liquid Chromatography
HREIMS - High Resolution Electron Ionization Mass Spectrometry
IBD - Irritable Bowel Diseases
IC$_{50}$ - Half Inhibitory Concentration
IR - Infrared
LDH - Lactate Dehydrogenase
LMA - Low Melting Point Agarose
MDA - Malondialdehyde
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MPLC - Medium Pressure Liquid Chromatography
NMR - Nuclear Magnetic Resonance
hrs - Hours
HBBS - Hanks' Balanced-Salt Solution
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PBS - Phosphate Buffered Saline
P-gp - P-glycoprotein
PMSF - Phenylmethanesulphonylfluoride
ROS - Reactive Oxygen Species
SOD - Superoxide Dismutase
TBA - Thiobarbituric Acid
TBARS - Thiobarbituric Acid Reactive Substances
TCA - Trichloroacetic Acid
TLC - Thin Layer Chromatography
TTX - Tetrodotoxin
1.0 INTRODUCTION

1.1 Ayurvedic Medicine

*Boerhaavia diffusa* is a medicinal plant widely used in the Ayurvedic medicine (Lad, 1999).

Ayurveda is an ancient traditional medical system of health care of the Veda civilization, flourishing in India many thousand years ago (Figure 1). The term “AYURVEDA” means “Knowledge or Science of Life” (from “Ayus” meaning “life” and “Veda” meaning “acquaintance”, “science”) in Sanskrit. In fact, Ayurveda focuses on the physical, spiritual and mental aspects of an individual. It supports and nourishes all the beneficial values of life and, what is more important, this medical practice is not in discord with any other medicinal science.

Ayurveda uses the balancing power of Nature, the power that every plant
Figure 1. Ayurveda system
contains, in order to correct the imbalances which are at the root of any
disease.

The fundamental nature of the Ayurvedic medical system is based on the
harmony that exists between the microcosm and the macrocosm. This notion
was applied to the body by the Ayrvedic physicians as they considered that the
body was composed of the same elements as the universe was created (earth,
fire, water, sky and air). According to the teachings of Ayurveda, most of the
body activities are governed by the three basic body energy control principles
known as “doshas” (humour) in Sanskrit. Each dosha is made up of one or two
of the five basic elements and each has a particular relationship to the body
functions. When the doshas are perfectly balanced, the result is the health with
the invaluable energy, but when this delicate equilibrium is disturbed (as it
happens if the body, the mind and the spirit are not in coordination), either
bodily or mental suffering develops. Each of these three doshas coordinates a
series of functions as follows:
• DOSHA VATA - It represents the air and ether principles of the movement and the activity. Dosha Vata controls all the activities of the nervous system and presides the functions of the respiratory and circular systems, the locomotion apparatus and the excretory organs;

• DOSHA PITTA - It represents the fire and water principles of the transformation and the production of energy and heat. Dosha Pitta controls the digestive and metabolic functions through the mechanism of the hormonal regulation; it also uses bile to direct digestion and hence metabolism into the venous system;

• DOSHA KAPHA - It represents the water and earth principles of the cohesion and the structure. Dosha Kapha preserves the integrity of the body tissue by controlling the immune system. It governs the equilibrium of the fluids at the cellular and tissue levels. This principle relates to mucus, lubrication and is the carrier of nutrients into the arterial system promoting the increased balance of the body.
The balanced interaction of the three doshas concurs the integrated and efficient operations of all the body parts and the psychological and physical health is experienced; on the contrary, when one or more of the three doshas influences are excessive or deficient the balance is disturbed and a disease is developed.

It is apparent that the Ayurvedic medicine, although it was created and practiced several thousand years ago, possesses the anatomic and functional conceptions of the human body. However, not only has it become actual nowadays, but it has turned out to be evolutionary because of its holistic vision of the structures and application.

The Ayurvedic medicine predominantly uses plant drugs, and therefore, it can be defined as the phytotherapy comprehended in a very sophisticated way. One of the most typical exemplary plants of the Ayurvedic medicine is Boerhaavia diffusa Linn (Lad, 1999) (Figure 2).

1.2 Boerhaavia diffusa
Figure 2. *Boerhaavia diffusa*
1.2.1. Botany

The plant was named in honour of Hermann Boerhaave, a famous Dutch physician of the 18th century (Chopra, 1969).

*Boerhaavia diffusa* (Spreading Hogweed in English), belonging to the family of the Nyctaginaceae, is mainly a diffused perennial herbaceous creeping weed of India (known also under its traditional name as *Punarnava*) and of Brazil (known as *Erva tostão*). *Boerhaavia diffusa* is up to 1 m long or more, having spreading branches. The stem is prostrate, woody or succulent, cylindrical, often purplish, hairy, and thickened at its nodes. The leaves are simple, thick, fleshy, and hairy, arranged in unequal pairs, green and glabrous above and usually white underneath. The shape of the leaves varies considerably ovate - oblong, round, or subcordate at the base and smooth above.

The margins of the leaves are smooth, wavy or undulate. The upper surface of the leaves is green, smooth and glabrous, whereas it is pinkish white and hairy beneath. The flowers are minute, subcapitate, present 4-10 together in small
bracteolate umbrellas, mainly red or rose (Figure 3), but the white varieties are also known (Figure 4). The achene fruit is detachable, ovate, oblong, pubescent, five-ribbed and glandular, anthocarpous and viscid on the ribs (Thakur et al., 1989). The seeds germinate before the onset of the monsoon. The plant grows profusely in the rainy season and mature seeds are formed in October-November. Due to its sticky nature, the plant gets stuck on the clothes of humans and on the legs of animals, which helps in its dispersal from one place to another. It has a large root system bearing rootlets. The tap root is tuberous, cylindrical to narrowly fusiform, conical or tapering, light yellow, brown or brownish grey (Figure 5). It is thick, fleshy and very bitter in taste (Capasso et al., 2000).

1.2.2 Ethnobotanical Use

Various parts of Boerhaavia diffusa are used for the treatment of numerous disorders in a different parts of India. In Purulia (West Bengal), the tribes eat this plant as a vegetable. Boerhaavia leaves are cooked and eaten in Assam,
**Figure 3.** *Boerhaavia diffusa* flowers

**Figura 4.** *Boerhaavia diffusa* flowers
Figure 5. *Boerhaavia diffusa* roots
eicosanoids, stearic and ursolic acids (Mishra and Tiwari, 1971), serine, liirodendrin (Aftab et al., 1996) carbohydrates, proteins, glycoproteins, etc.

The most interesting metabolites from the therapeutic point of view are the rotenoids (known as boeravinones A - F) (Figure 6) (Misra and Tewari, 1971; Jain and Khanna, 1989; Kadota et al., 1989; Lami et al., 1990; 1992).

1.2.3 Biological Activity

1.2.3.1 Traditional Medical Use

The root, leaves, aerial parts or the whole plant of *Boerhaavia diffusa* have been employed for the treatment of various disorders in the Ayurvedic herbal medicine (daily used by millions of people in India, Nepal, Sri Lanka and indirectly through it being the major influence on Unani, Chinese and Tibetan medicines). The root is mainly used to treat gonorrhea, internal inflammation of all kinds, dyspepsia, oedema, jaundice, menstrual disorders, anaemia, liver, gallbladder and kidney disorders, enlargement of spleen, abdominal pain,
Boeravinone A  
Boeravinone B  
Boeravinone C  
Boeravinone D  
Boeravinone E  
Boeravinone F  

Figure 6. Main rotenoids (known as boeravinones A-F) present in the root of *Boerhaavia diffusa*
abdominal tumours, and cancers (Kirtikar and Basu, 1956), then as a diuretic (documented in Indian Pharmacopoeia - Chopra, 1969), digestive aid, laxative and a menstrual promoter. The root powder, when mixed with *mamira (Thalictrum foliolosum)*, is used to treat eye diseases. It cures corneal ulcers and night blindness (Gupta et al., 1962), and helps restore virility in men. People in tribal areas use it to hasten childbirth (Shah et al., 1983). The juice of *Boerhaavia diffusa* leaves serves as a lotion in ophthalmia. It is also administered orally as a blood purifier and to relieve muscular pain (CSIR, 1988).

1.2.3.2 Pharmacological and Clinical Properties

The first pharmacological studies have demonstrated that the root of *Punarnava* exhibits a wide range of properties: anti-inflammatory (Bhalla et al., 1968, 1971), diuretic (Gaitonde et al., 1974), laxative (Chopra et al. 1956), anti-urethritis (Nadkarni, 1976), anticonvulsant (Adesina, 1979), antinematodal (Vijayalakshmi et al., 1979), antifibrinolytic (Jain and Khanna, 1989),
antibacterial (Olukoya et al., 1993), antihepatotoxic (Mishra, 1980; Chandan et al., 1991; Rawat et al., 1997), anthelmintic, febrifuge, antileprotic, anti-asthmatic, antiscabby and antistress activities. An aqueous extract of thinner roots of *B. diffusa* at a dose of 2 mg/kg exhibited the remarkable protection of various enzymes such as serum glutamic-oxaloacetic transaminase, serum glutamic-pyruvic transaminase, and bilirubin in serum against hepatic injury in rats (Rawat et al., 1997). Maximum diuretic and anti-inflammatory activities of *Punarnava* have been observed in samples collected during the rainy season. Due to the combination of these two activities, *Punarnava* is regarded therapeutically highly efficacious for the treatment of renal inflammatory diseases and common clinical problems such as nephritic syndrome, oedema, and ascites developing at the early onset of the liver cirrhosis and chronic peritonitis. The root is used to treat other renal ailments (calculations and cystitis), seminal weakness and blood pressure (Gaitonde et al., 1974) and as a diuretic (Singh et al., 1992; Anand, 1995). It is also used in the treatment of stomach ache, anaemia, cough, and cold, and as a
diaphoretic, laxative, expectorant and a potent antidote for snake and rat bites (Chopra et al., 1956). *Punarnava* is useful in the treatment of nephritic syndrome (Singh and Udupa, 1972), hepatitis, gall bladder abnormalities and urinary disorders (Mudgal, 1975; Cruz, 1995). The flowers and seeds are used as a contraceptive (Chopra et al., 1956).

The plant was reported to be efficient for the treatment of the abdominal tumours and cancers and was proved to be useful as a haematic and as a growth promoter with the children fed with milk fortified with the plant drug. In the form of a powder or an aqueous decoction, the plant drug was proved to be beneficial in the treatment of nephritic syndrome and compared well with corticosteroids. It was also demonstrated that the drug decreased the albumin urea, increased the serum protein and lowered serum cholesterol level (Ramabhimaiah et al., 1984).

Singh and Udupa (1972) reported that the dried root powder showed curative efficiency when administered orally for one month to the children or adults suffering from the helminth infection. The patients became worm-free
within five days of the treatment. The drug, singly or in combination with other
drugs, was found to be efficient in liver disorders, gastrointestinal disorders, heart
diseases (hypertension, angina, cardiac failure, etc.), respiratory tract infections,
leukorrhea, spermatorrhea, etc. The purified glycoprotein from *B. diffusa*
exhibited strong antimicrobial activity against RNA bacteriophages (Awasthi and
Menzel, 1986).

Chakraborti and Handa (1989) also reported a hepatoprotective activity of
the aerial parts of *B. diffusa*. The hepatoprotective activity of the *B. diffusa*
root was demonstrated by Rawat (1987) and by Chandan (1991) too. These
investigators found that the watery extract from the root of *B. diffusa*
minimised the toxic effects generated by the CCl₄ and the tioacetamide in the
liver. Further experimental studies also evidenced a beneficial activity of the
*Punarnava* root for the treatment of the jaundice (Singh and Pandey, 1980;
Gopal and Shah, 1985).
The treatment with the watery extract from the root of *B. diffusa* induced leucocytosis with predominant neutrophils, associated to the phagocytosis ability and it was bactericidal to the neutrophils and the macrophages (Mungantiwar et al., 1997).

The recent study carried out by Pari et al. (2004) demonstrated that the leaves of *B. diffusa* reduced the levels of glucose in the blood increasing the insulin release from the β cells of pancreas. The watery extract of *B. diffusa* was proved to possess protective abilities to the rodents suffering from the peritonitis induced by *Escherichia coli* (Hiruma-Lima et al. 2000).

It was evidenced that the leaves and root possessed antifibrinolitic and anti-inflammatory activities (Hiruma-Lima, 2000). In the recent study, led by Mehrotra et al. (2002) was reported that the etanolic extract of *B. diffusa* showed a significant immunosuppressive activity on human cells and on murine cells as well.
Toxicological studies conducted on *B. diffusa* demonstrated the absence of teratogenic and mutagenic effects (Singh et al., 1991).
2.0 RESEARCH OBJECTIVES

*Boerhaavia diffusa* is a widely used plant in Ayurvedic medicine for the treatment of several illnesses such as inflammatory and gastrointestinal ones.

Despite its extensive use in folk medicine a few phytochemical studies aimed at analyzing the effect of this plant have been performed until now.

Therefore the purpose of the study was:

1. to estimate the effect of a methanolic root extract of *Boerhaavia diffusa* on the intestinal motility;

2. to evaluate a potential antioxidant effect of the methanolic root extract of *Boerhaavia diffusa*;

3. to estimate a genoprotective effect of the methanolic root extract of *Boerhaavia diffusa*;

4. to isolate and to identify the active metabolites responsible of the pharmacological properties of the *Boerhaavia diffusa* methanolic root extract;
5. to perform toxicological study *in vitro* on the methanolic root extract of *Boerhaavia diffusa*.

In addition, considering the use of *Boerhaavia diffusa* in the adjuvant treatment of several tumours, another aim of the thesis is:

6. to analyse the effect of isolated compounds from the methanolic root extract of *Boerhaavia diffusa* on the Breast Cancer Resistance Protein Activity (BSPR).
3.0 MATERIALS AND METHODS

3.1 Plant Material

The samples of the root of *Boerhaavia diffusa* L. (Nyctaginaceae), collected in Bangalore (India), were kindly provided by Dr. Carlo Sessa, Milan. A voucher specimen (N° 22.02) is deposited in the Carlo Sessa Herbarium (Viale Gramsci 212, 20099 Sesto S. Giovanni, Milano, Italy). The rootstocks and root (1.0 kg) were extracted (3 × 3 L) with methanol at room temperature for 1 hr. The evaporated pooled extract left a brown substance (44.4 g) that was then subjected to the modified Kupchan partition scheme which is shown in the adequate place of the study (Kupchan et al., 1973).

In order to test the intestinal motility, the methanolic extract of the *Boerhaavia diffusa* root was dissolved in DMSO water (50% v/v). The subsequent dilutions were carried out in Krebs solution (see page 29 for composition).
To test the antioxidant and genoprotective activity and to perform toxicological studies, the methanolic root extract of *Boerhaavia diffusa* was dissolved in DMSO water (50% v/v) to obtain a stock solution of 1 mM (Hanks’ Balance Salt Solution) (MDA assay) or in the cell culture media (MTT and Comet assays).

### 3.2 Studies on Intestinal Motility

#### 3.2.1 Substances and Drugs

The employed substances to test the intestinal motility were: nifedipine, ethylenediaminetetraacetic acid (EDTA), cyclopiazonic acid, acetylcholine chloride, histamine and barium chloride (Sigma, Milan, Italy). All chemicals and reagents employed in this thesis were of analytical grade.

Acetylcholine chloride, barium chloride (BaCl₂) and histamine were dissolved in distilled water; nifedipine and cyclopiazonic acid were dissolved in dimethyl sulfoxide (DMSO) while the EDTA was dissolved in distilled water to the constant temperature of 60°C.
3.2.2 Bioassay-Guided Isolation of the Active Compounds

These experiments were done in collaboration with Prof. Ernesto Fattorusso and Prof. Orazio Taglialatela-Scafati (Department of Chemistry of Natural Products, Faculty of Pharmacy, University of Naples Federico II).

A small part of the crude methanolic extract (13.4g), obtained from *Boerhaavia diffusa* root, was fractioned following the modified Kupchan method (Kupchan et al.,1973). The methanolic extract was dissolved in MeOH-H₂O (9:1) and then partitioned against *n*-hexane (3 × 500 ml) to yield an apolar extract weighing 1.55 g. Subsequently, the water content of the hydromethanolic phase was adjusted to 20% (v/v) and 40% (v/v) and the solutions were partitioned against carbon tetrachloride (CCl₄, 3 × 500 ml) and chloroform (CHCl₃, 3 × 500 ml), respectively, affording the CCl₄ (0.56 g) and CHCl₃ (0.90 g) extracts. Finally, all the MeOH was evaporated from the hydromethanolic layer, and the water solution thus obtained was partitioned against *n*-BuOH to yield the butanol (1.32 g) and water (7.0 g) extracts.
The preliminary studies of the intestinal motility showed that the CCl₄ and
the CHCl₃ fractions were active and, therefore, they were further separated.

The CCl₄ was chromatographed by MPLC (Medium Pressure Liquid
Chromatography) on the silica gel (230-400 mesh) column (750 × 25 mm),
using a linear gradient system (400 ml for each solvent) from n-hexane to
EtOAc to MeOH-EtOAc (1:1). The MPLC chromatography is technically
based on the employment of pumps in order to push the eluent into the
column. The obtained pressure was between 2 and 4 atmospheres during the
separate processes. In such a way it was possible to employ the stationary
phases with a rather fine granulometry, therefore, a good degree of the
complex solid matrix resolutions. Following the pharmacological profile the
series of purifications were carried out. All the obtained fractions were
subjected to biological studies and those more active were further separated by
HPLC.

The tetrachloruric fraction (CCl₄ fraction) was first separated:
the first fraction (\(n\)-hexane-EtOAc, 8:2) was purified by HPLC on an analytical column (250 × 4.6 mm) using \(n\)-hexane-EtOAc (85:15) as an eluent, flow rate 1.0 ml/min, affording 2-\(O\)-methylabronisoflavone (9, 3.6 mg, \(t_R = 13.2\) min) and coccineone E (8, 7.9 mg, \(t_R = 14.1\) min). The second fraction (hexane/EtOAc 7:3) was purified by HPLC on analytical column (250 × 4.6 mm) using hexane/EtOAc 75:25 as an eluent, flow rate 1.0 ml/min, obtaining two new compounds, denominate by us, boeravinone G (5, 2.1 mg, \(t_R = 16.5\) min) and boeravinone H (4, 3.8 mg, \(t_R = 14.5\) min), and the known compound 6-\(O\)-demethylboeravinone H (3, 1.1 mg, \(t_R = 20.5\) min) (Figure 7). The third fraction (hexane/EtOAc 6:4) was purified by HPLC on analytical column (250 × 4.6 mm) using hexane/EtOAc 65:35 as eluent, flow rate 1.0 ml/min, obtaining the known compounds boeravinone D (1, 7.5 mg, \(t_R = 15.5\) min) and boeravinone E (2, 6.3 mg, \(t_R = 19.5\) min) (Figure 7).

The fourth fraction (\(n\)-hexane-EtOAc, 1:1) was purified by HPLC on an analytical column (250 × 4.6 mm) using \(n\)-hexane-EtOAc (55:45) as eluent,
Figure 7. Rotenoids isolated from the methanolic root extract of *Boerhaavia diffusa* and tested on intestinal motility, antioxidant, genoprotective and cytotoxic activities.
flow 1.0 ml/min, affording the known compound 7 (4,11,12a-trihydroxy-9-methoxyrotenone, 1.8 mg, \( t_R = 12.5 \) min). The fifth fraction \((n\)-hexane-EtOAc, 4:6\) was purified by HPLC on an analytical column \((250 \times 4.6 \text{ mm})\) using \(n\)-hexane-EtOAc (4:6) as eluent, flow rate 0.8 ml/min, yielding boeravinone C (6, 5.5 mg, \( t_R = 12.9 \) min). Finally, the sixth fraction \((n\)-hexane-EtOAc, 3:7\) was purified by HPLC on analytical column \((250 \times 4.6 \text{ mm})\) using \(n\)-hexane-EtOAc (35:65) as an eluent, flow rate 0.8 ml/min, affording another new compound - 6,10,11-trihydroxy-9-methoxyrotenone (12, 1.5 mg, \( t_R = 8.8 \) min).

The CHCl\(_3\) extract was chromatographed by MPLC on a silica gel (230-400 mesh) column \((750 \times 25 \text{ mm})\), using a linear gradient system (400 ml for each solvent) from \(n\)-hexane-EtOAc (7:3) to EtOAc to EtOAc-MeOH (1:1). The fraction eluated with EtOAc-\(n\)-hexane (7:3) was further purified by HPLC (EtOAc-\(n\)-hexane, 6:4, flow rate 0.8 ml/min) yielding boeravinone F (10, 2.2 mg, \( t_R = 7.9 \) min) and coccineone B (11, 2.1 mg, \( t_R = 9.0 \) min) in the pure state (Figure 7).
**Boeravinone G (5):** pale yellow amorphous solid; yield: 2.1 mg, purity: 98% (by HPLC, RI detection, and NMR); \([\alpha]_D^{25} 0^\circ (c 0.01 \text{ in CHCl}_3); \) IR (KBr): \(\nu_{\text{max}} 3250, 1648, 1618 \text{ cm}^{-1}; \) UV (CH\(_3\)CN): \(\lambda_{\text{max}} 343, 300, 279 \text{ nm (log}\varepsilon: 3.70, 4.30, \text{ and } 4.50, \text{ respectively).} \) \(^1\)H (500 MHz) and \(^{13}\)C (125 MHz) NMR spectra (CDCl\(_3\)): see Appendix. ESIMS (negative ions): \(m/z 341 \text{ [M-H]}^-\). EIMS (70 eV): \(m/z 342 \text{ [M]}^+ (40), 311 \text{ [M-OCH}_3\]}^+ (100). HREIMS: found \(m/z 342.0731 \) (calcd. for C\(_{18}\)H\(_{14}\)O\(_7\), \(m/z 342.0740\)).

**Boeravinone H (4):** pale yellow amorphous solid; yield: 3.8 mg, purity: 98% (by HPLC, RI detection, and NMR); \([\alpha]_D^{25} 0^\circ (c 0.01 \text{ in CHCl}_3); \) IR (KBr): \(\nu_{\text{max}} 3248, 1648, 1618 \text{ cm}^{-1}; \) UV (CH\(_3\)CN): \(\lambda_{\text{max}} 345, 302, 279 \text{ nm (log}\varepsilon: 3.70, 4.30, \text{ and } 4.50, \text{ respectively).} \) \(^1\)H (500 MHz) and \(^{13}\)C (125 MHz) NMR spectra (CDCl\(_3\)): see Appendix. ESIMS (negative ions): \(m/z 355 \text{ [M-H]}^-\). EIMS (70 eV): \(m/z 356 \text{ [M]}^+ (40), 325 \text{ [M-OCH}_3\]}^+ (100). HREIMS: found \(m/z 356.0908 \) (calcd. for C\(_{19}\)H\(_{16}\)O\(_7\), \(m/z 356.0896\)).

**6,10,11-Trihydroxy-9-methoxyrotenone (12):** pale yellow amorphous
solid; purity 98% (by HPLC and NMR); \([\alpha]_D^{25} 0 \ (c \ 0.01, \text{MeOH})\); UV (CH\(_3\)OH) \(\lambda_{\text{max}}\) 343, 281 nm (log\(e\) 3.70 and 4.50, respectively); UV (CH\(_3\)OH + AlCl\(_3\)) \(\lambda_{\text{max}}\) 378, 284 nm; UV (CH\(_3\)OH + AlCl\(_3\)/HCl) \(\lambda_{\text{max}}\) 381, 285 nm; UV (CH\(_3\)OH + NaOAc/H\(_3\)BO\(_3\)) \(\lambda_{\text{max}}\) 352, 285 nm; IR (KBr) \(\nu_{\text{max}}\) 3270, 1650, 1616 cm\(^{-1}\); \(^1\)H NMR (500 MHz, CD\(_3\)OD): see Appendix. ESIMS (negative-ion) \(m/z\) 327 [M-H]\(^-\); EIMS (70 eV): \(m/z\) 328 [M]\(^+\) (30), 311 [M-OH]\(^+\) (100); HREIMS found \(m/z\) 328.0576 (calcd for C\(_{17}\)H\(_{12}\)O\(_7\), \(m/z\) 328.0583).

The structure of these molecules was identified on the basis of the comparison of their spectral data (NMR) with those reported in the literature.

### 3.2.3 Animals

Guinea pigs (New Zealand, weighing 250-400 g), supplied by Harlan Italy, Corezzana, MI, Italy, were used after 1 week of acclimation (light-dark cycles, for 12 hrs, temperature 23 ± 2°C; humidity 60%). The rodents had free access to water and food (supplied by the Eat Mucedola company, Settimo Milanese, Italy). All experiments on the animals complied with the Italian D.L. no. 116.

3.2.4 Guinea Pig Ileum Isolation

Guinea-pigs were killed by asphyxiation with CO₂ and the segments (2–3 cm) of the terminal ileum were removed, flushed of luminal contents and placed in the Krebs solution (composition in mM: NaCl 119, KCl 4.75, KH₂PO₄ 1.2, NaHCO₃ 25, MgSO₄ 1.5, CaCl₂ 2.5, and glucose 11). The isolated ileum parts were cut perpendicularly along the longitudinal muscles to obtain segments of 1 cm length. The ileum segments were suspended in a bath (37°C) containing the Krebs solution which was aerated (95% O₂ : 5% CO₂). The tissues were connected to a isotonic transducer (load 0.5 g) connected to a PowerLab data-acquisition system (Ugo Basile, Comerio, Italy).

3.2.5 Contractions Induction

At the beginning of each experiment, the ileum segments was stimulated with acetylcholine (10⁻³ M) in order to obtain a maximal contraction (100%
contraction). After a minimal one-hour of the equilibration period, the tissues were subjected to (i) the electrical field stimulation (EFS, 10 Hz for 0.3 s, 100 mA, 0.5 ms pulse duration using a multiplexing pulse booster by Ugo Basile, Milan, Italy) via a pair of platinum electrodes (situated at the distance of 1.5 cm) placed around the intestine or (ii) stimulated with exogenous spasmogens, namely acetylcholine (10^{-6} M), BaCl_2 (10^{-3} M) and histamine (10^{-6} M). The concentrations of the agonists gave a contractile response which was similar in amplitude to that of EFS. Acetylcholine, BaCl_2 and histamine, added to the bath, were left in contact with the tissue for 30 sec. After recording the contractions, the ileum was washed with the Krebs solution in order to remove the effects of the exogenous spasmogens. After at least three stable control contractions, the responses were repeated in the presence of increasing (non-cumulative) concentrations of _B. diffusa_ methanolic root extract (1-1000 \mu g/ml) added 20 min before the contacting stimulus (after washing the tissue). The preliminary experiments showed that a 20 min contract time was
sufficient for the *B. diffusa* methanolic root extract to achieve the maximal inhibitory effect.

The conditions of EFS were selected so that the contractile responses were similar in amplitude to those of acetylcholine, histamine and BaCl\(_2\). The stable and reproducible contractions for a period of 4 hrs were obtained, with stimulations every 20 min. After the stable control contractions evoked by EFS of the cholinergic nerves had been registered, the responses were observed in the presence of the increased cumulative concentrations of *B. diffusa* methanolic root extract (1-1000 µg/ml). The contact time for each concentration was 20 min.

In the preliminary experiments, the effect of tetrodotoxin (TTX; 3 ×10\(^{-7}\) M, contact time: 20 min) was evaluated on both exogenous spasmogens - and electrical - induced contractions. The electrical stimulation of the guinea pig ileum gave a contractile response that was abolished by TTX (3 × 10\(^{-7}\) M) (data not shown). By contrast, the contractions induced by acetylcholine,
histamine, and BaCl₂ were not modified by TTX (3 × 10⁻⁷ M) (data not shown).

In some experiments, the effect of *B. diffusa* methanolic root extract on acetylcholine-induced contractions was performed in the presence of nifedipine (10⁻⁶ M - a blocker of L-type Ca²⁺ channels), cyclopiazonic acid (10⁻⁵ M - a potent and specific inhibitor of the sarcoplasmic reticulum Ca²⁺-ATPase in smooth muscle), and ethylenediaminetetraacetic acid (EDTA, 10⁻³ M - a calcium chelator) (contact time: 20 min). The concentrations of nifedipine, cyclopiazonic acid and EDTA were selected on the basis of previous studies (Uyama et al., 1992; Izzo et al., 1999; Aronsson and Holmgren, 2000; Lis-Balchin and Hart, 2002). When given alone (i.e., in the absence of *B. diffusa* methanolic root extract), nifedipine (10⁻⁶ M), EDTA (10⁻³ M) and cyclopiazonic acid (10⁻⁵ M) significantly (*p* <0.01) reduced (% reduction: nifedipine: 47.3 ± 2.9, EDTA: 49.7 ± 5.2, cyclopiazonic acid: 52.3 ± 5.8, *n* = 8 for each drug) the contractions induced by acetylcholine.
3.3 Antioxidant Activity

3.3.1 Substances and Drugs

The used substances to test antioxidant activity were: sodium chloride, dimethyl sulfoxide, sodium hydroxide, malondialdehyde, trypsin, trypan blue, methanol, hydrogen peroxide, ferrous sulphate, Tris, Triton-X, 5,5-dimethyl-1-pyrroline N-oxide (DMPO), sucrase, oxidase/peroxidase, O-dianisidine, 2’,7’-dichlorfluorescein-diacetate (H$_2$DCF-DA) and 1,1,3,3-tetramethoxypropane which were purchased from Sigma (Milan, Italy). All the reagents for the cellular cultures were supplied by the Microtech Research Products (Naples, Italy). EDTA, sodium chloride, ethidium, sodium hydroxide and Tris were dissolved in distilled water. The ferrous sulphate was dissolved in HBBS (Hanks' Balanced-Salt Solution).

3.3.2 Bioassay-Guided Isolation of the Active Compounds

These experiments were done in collaboration with Prof. Ernesto Fattorusso and Prof. Orazio Taglialatela-Scafati (Department of Chemistry
of Natural Products, Faculty of Pharmacy, University of Naples Federico II).

A small part of the crude methanolic extract (13.4g), obtained from B. *diffusa* root, was fractioned following the modified Kupchan method (Kupchan et al., 1973), as was explained on page 23. The most antioxidant fraction (CCl₄) was chromatographed by MPLC on silica gel (230-400 mesh) column (750 × 25 mm), using a linear gradient system (400 ml for each solvent) from *n*-hexane to EtOAc to MeOH-EtOAc (1:1). The obtained fractions were pooled on the basis of their TLC behavior to afford 13 fraction called BOE-1 to BOE-13. All these fractions were subjected to the ESR assay and the most active, BOE-4, BOE-6, BOE-8 and BOE-9 were further separated by HPLC. Both BOE-4 (*n*-hexane-EtOAc, 8:2) and BOE-6 (*n*-hexane-EtOAc, 7:3) fractions were purified by HPLC on an analytical column (250 × 46 mm) using *n*-hexane-EtOAc 75:25 as eluent (flow rate 1.0 ml/min) and afforded as the main component boeravinone D (1, Figure 11, 6.4 mg). The BOE-8 fraction (eluated with hexane/EtOAc 6:4) was purified by HPLC on an analytical column using
hexane/EtOAc 7:3 as an eluent, flow rate 1.0 ml/min, obtaining boeravinone G (5, Figure 11, 7.5 mg). Finally, the BOE-9 fraction (eluated with hexane/EtOAc 1:1) was purified by HPLC on an analytical column using hexane/EtOAc 6:4 as an eluent, flow rate 1.0 ml/min, obtaining boeravinone H (4, Figure 11, 5.2 mg). The structure of these molecules was identified on the basis of the comparison of their spectral data with those reported in the literature.

3.3.3 Cell Culture

Human colon adenocarcinoma cells (Caco-2 cells) were used for the experiments. Caco-2 cells were cultured in Roux flasks as monolayers in DMEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (foetal bovine serum), 1% non-essential amino acids and 100 U/l penicillin/streptomycin, 1% L-glutamine 2mM and 2.5% HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). The cells were cultured for 7 days (~ 70% confluence) at 37°C in the humidified 5% CO₂ and 95% air and
the culture medium was replaced every 2 days. After washing in PBS (phosphate buffered saline), the cells were trypsinized with 0.25 % trypsin-EDTA at 37°C for 5 min, centrifuged at 1200 rpm/3 min and re-suspended in the appropriate medium. The viability of the cells was estimated by trypan blue staining. The Caco-2 cells were purchased from the American Type Cultures Collection (ATCC, Milan, Italy).

3.3.4 Electronic Spin Resonance Spectrometer (ESR)

The effect of the methanolic root extract of *B. diffusa* and of its fractions were tested on the formation and the transformation of the hydroxyl radicals obtained by the Fenton reaction [0.2 ml of H$_2$O$_2$ (10 mM), 0.2 ml of FeCl$_2$ x 4 H$_2$O (10 mM) and 0.2 ml of 5,5-dimethyl-1-pyrroline N-oxide (DMPO, 0.3 M) used as a spin trap (blank)]. The hydroxyl radical production was detected by the ESR spectrometer Bruker 3000E (Rheinstetten, Germany) with the following settings: field modulation 100 kHz, modulation amplitude 0.512 G, receiver gain $2 \times 10^5$, time constant 81.92 ms, conversion time 163.84 ms,
center field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW, temperature 23°C (Čanadanović-Brunet et al., 2005). The ESR spectrometer is made of a source of electromagnetic radiations (a cavity located between the poles of an electromagnet where the sample under the investigation is put), an electromagnet (where the generation of the oscillating magnetic field is produced) and an apparatus capable to record the quantity of radiations absorbed from the samples (Figures 8a and 8b).

The influence of the methanolic root extract of \textit{B. diffusa} and its fractions (constituents) on the formation and transformation of hydroxyl radicals were investigated by adding the \textit{B. diffusa} methanolic root extract to the Fenton reaction system in the range of the concentrations 0.10-5 mg/ml and 0.01-1 mg/ml for the \textit{B. diffusa} methanolic root extract fractions, respectively.

The methanolic root extract of \textit{B. diffusa} of the 0.1-5 mg/ml concentrations was introduced in the cavity of the ESR spectrometer in which the Fenton
Figure 8: ESR Spectrometer (a) and ESR scheme (b)
reagent had previously been inserted. The ESR spectra were recorded after 5 min of the beginning of the reaction under the following conditions: 100 KHz intensity, amplitude 0.512 G, constant time 81.92 ms, time of conversion 163.84 ms, curve amplitude 100.00 G, frequency band 9.64 GHz, power 20 mW, temperature 23°C (Cetkovic et al., 2004). The antiradical activity (AA) of the extracts was expressed as a percentage of the height reduction of the peaks height under the given formula:

\[
AA = 100 \times \left(\frac{I_{h_0} - h_x}{h_0}\right)
\]

Where:

\[h_0=\text{height of the peak before adding the extract (the control)}\]

\[h_x=\text{height of the peak after adding one of the extracts (investigated probe)}\]

### 3.3.5 Sucrase - Isomaltase Activity Determination

Sucrase-isomaltase is the most studied brush border enzyme and it is a good differentiation marker. It is found on the luminal surface of the Caco-2 cells and in small intestinal cells. The activity of sucrase-isomaltase is low during
the proliferation phase and increases once the confluence is reached. The sucrase-isomaltase activity was detected using Dahlqvist method (1970). The Caco-2 cells were seeded in 6-well plates at the density of 4.0 x 10^5 cells/well in DMEM supplemented with 10% FBS at 37°C for 3, 5, 7, 10, 12 and 14 days. After washing in PBS, the cells were collected with a lysis buffer (50mM Tris-HCl pH=7.4, 0.25 % sodium deoxicolate, 150 mM NaCl, 1 mM EGTA, 1 mM NaF, 1% NP-40, 1 mM PMSF, 1 mM Na_3VO_4 and a mixture of protease inhibitors) (Roche Diagnostics, Mannheim, Germany). The cells were subsequently centrifuged at 13000 rpm for 10 min at 4°C and an aliquot of the extract was used for the proteic detection with the Bradford method (Bio-Rad, CA). An aliquot of proteic extract was incubated with a sucrase solution for 1 hr at 37°C. Then the samples were incubated with a glucose oxidase/peroxidase and O-dianisidine to allow sucrase-isomaltase to convert the glucose and fructose in orange-brown chromophor, which was evaluated spectrophotometrically at the wavelength (λ) of 430 nm.
3.3.6 TBARS Assay

Malondialdehyde (MDA) is a product of the lipid peroxidation. It is a powerful toxic member which causes the “cross-linkage” of proteins and enzymes, altering their biological functions. The effect of the *B. diffusa* methanolic root extract (30-300 µg/ml) on lipid peroxidation was assessed by measuring the cytosolic levels of thiobarbituric acid reactive substances (TBARS) according to Ohkawa et al. (1979). The Caco-2 cells were seeded in the 6-well plates at the density of $3.0 \times 10^6$ per well and led to the differentiation (evaluated with sucrase-isolmaltase assay). The differentiated cells were treated with the *B. diffusa* methanolic root extract (30-300 µg/ml) or boeravinone G (0.0001-0.001 µg/ml) for 24 hrs and then washed with PBS and incubated with the Fenton reagent ($\text{H}_2\text{O}_2/\text{Fe}^{2+}$ 1 mM) for 3 hrs at 37°C. After the incubation, the cells were washed and scraped in PBS. The cells were lysed by six cycles of freezing and thawing in PBS and then centrifuged at 13000 rpm for 10 min at 4°C. The 300 µl of 10% (w/v) trichloroacetic acid (TCA)
was added to 150 µl of cellular lysate and, after centrifugation at 13000 rpm for 10 min, 0.67% (w/v) of thiobarbituric acid was added to the supernatant and the mixture was heated at 80°C for 30 min. After cooling, the malondialdehyde (MDA) formation was determined spectrophotometrically with the wavelength (λ) of 532 nm using a multiwell plate reader (Beckman DU62). The quantification of TBARS was determined by comparing its absorption curve to the standard curve of the malondialdehyde (MDA) equivalents generated by the acid catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. The results were presented as µmol of MDA/mg of the cell protein determined by the Bradford assay (Bio-Rad).

3.3.7 Intracellular ROS Measurement

The generation processes of the intracellular reactive oxygen species (ROS) can be monitored using the fluorescence probe 2’;7’-dichlorfluorescein-diacetate (H₂DCF-DA) (Yokomizo et al., 2006). The H₂DCF-DA is a non-fluorescent permeant molecule that diffuses passively into cells, where the
acetate is cleaved by intracellular esterases to the non-fluorescent H$_2$DCF which thereby traps it within the cell. In the presence of intracellular ROS, H$_2$DCF is rapidly oxidized to the highly fluorescent 2’,7’-dichlorofluorescein (DCF). Therefore the DCF fluorescence intensity is paralleled to the amount of ROS formed intracellularly. For the experiments, the cells were plated in a 96 multiwell black plate (Corning, USA) at the density of 1×10$^4$ cells per well and led to the differentiation (evaluated with sucrase-isomaltase assay). The confluent Caco-2 cell monolayers were incubated for 24 hrs at 37°C with the B. diffusa methanolic root extract (30-300 µg/ml) or boerhavinone G (0.0001-0.001 µg/ml). The cells, were then, rinsed and incubated for 30 min with 200 µl of 100 µM DCFH-DA in HBSS containing 1% FBS. Finally, the cells were rinsed and incubated with the Fenton reagent (H$_2$O$_2$/Fe$^{2+}$ 2 mM) for 3 hrs at 37°C. The DCF fluorescence intensity was detected using a fluorescent microplate reader (Perkin-Elmer Instruments) at the excitation wavelength ($\lambda$) of 485 nm and the emission wavelength ($\lambda$) of 538 nm.
3.4 Genoprotective Assay

3.4.1 Substances and Drugs

The substances used to test genoprotective activity were: agarose, sodium chloride, sodium hydroxide, ethidium bromide, trypsin, trypan blue, hydrogen peroxide, ferrous sulphate, Tris and Triton-X which were purchased from Sigma (Milan, Italy). All the reagents for the cellular cultures were supplied by the Microtech Research Products (Naples, Italy). EDTA, sodium chloride, sodium hydroxide and Tris were dissolved in distilled water. Agarose and ethidium bromide were dissolved in PBS.

3.4.2 Bioassay-Guided Isolation of the Active Compounds

The isolation procedure is described on page 34.

3.4.3 Comet Assay

The presence of the DNA fragmentation was examined by the single cell gel electrophoresis (Comet assay), as described by Gill et al. (2005). The Caco-2 cells were seeded in the 25 cm² flasks at the density of the 4x10⁵ cells
and incubated with the *B. diffusa* methanolic root extract (30-300 μg/ml) at 37°C for 24 hrs. After the incubation with and without 75μM H₂O₂ challenge for 5 min on ice, the Caco-2 cells (1 × 10⁵ cells) were centrifuged at 1250 rpm for 5 min. The supernatant was discarded and the pellet was mixed with 85 μl of 0.85% low melting point agarose (LMA) in PBS. The cells were added to the previously prepared gels of 1% normal agarose (NMA). The gels on the frosted slides were maintained in the lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris and 1% Triton X-100, pH 10) at 4°C for 1 hr, and, then, electrophoresed in an appropriate buffer (300 mM NaOH, 1 mM Na₂EDTA, pH>12) at 26 V, 300 mA for 20 min. After running, the gels were neutralized in 0.4 M Tris–HCl, pH 7.5 (3 x 5 min washes) and stained with 20 μl of ethidium bromide (2 μg/ml) before scoring. The images were analyzed using a fluorescence microscope (Nikon) interfaced with the computer. The Komet 5.0 image analysis software (Kinetic Imaging) allowed to analyze and quantify the DNA damage by measuring the tail length. For each slide, 100
cells were scored. The positive (H₂O₂; 75 µmol/l) and negative (PBS) controls were included for all the experiments. The mean percentage of the DNA tail was calculated from 100 cells per gel (each sample in triplicate) and the mean of each independent experiment (n = 3) was used in the statistical analysis.

3.5 BCRP Mediated Inhibition Assay

3.5.1 Substances and Drugs

Dimethyl sulfoxide (0.5% final solution), mitoxantrone (5 µM), elaclidar (µM) and PBS were used.

3.5.2 Bioassay-Guided Isolation of the Active Compounds

These experiments were done in collaboration with Prof. Ernesto Fattorusso and Prof. Orazio Tagliatela-Scafati (Department of Chemistry of Natural Products, Faculty of Pharmacy, University of Naples Federico II).

A small part of the crude methanolic extract (13.4g), obtained from B. diffusa root, was fractioned following the modified Kupchan method (Kupchan et al., 1973), as was explain on page 22. For this part of the experiments the CCl₄
and CHCl₃ fractions were combined and then chromatographed by MPLC on a silica gel (230-400 mesh) column (750 × 25 mm), using a linear gradient system (400 ml for each solvent) from *n*-hexane to EtOAc to MeOH-EtOAc (1:1). All obtained fractions were subjected to the preliminary spectroscopic investigation and those apparently containing rotenoids were further separated by HPLC. The first fraction (*n*-hexane-EtOAc, 8:2) was purified by HPLC on an analytical column (250 × 4.6 mm) using *n*-hexane-EtOAc 85:15 as an eluent, flow rate 1.0 ml/min, affording coccineone E (8, 7.9 mg) (Figure 9). The second fraction eluated with hexane/EtOAc 7:3 was purified by HPLC on an analytical column using hexane/EtOAc 75:25 as an eluent, flow rate 1.0 ml/min, obtaining boeravinone G (5, 8.8 mg), boeravinone H (4, 6.4 mg), 6-*O*-demethylboeravinone H (3, 2.4 mg), boeravinone A (13, 12.3), and boeravinone B (14, 6.2 mg). The third fraction eluated with hexane/EtOAc 6:4 was purified by HPLC on an analytical column using hexane/EtOAc 65:35 as an eluent, flow rate 1.0 ml/min, obtaining boeravinone E (2, 8.4 mg). The
Figure 9. Rotenoids isolated from the methanolic root extract of *Boerhaavia diffusa* and tested on BCPR activity
fourth fraction eluted with \( n \)-hexane-EtOAc, 1:1, was purified by HPLC on an analytical column (250 × 46 mm) using \( n \)-hexane-EtOAc 55:45 as an eluent, flow rate 1.0 ml/min, affording the new compounds boeravinone I (15, 1.3 mg) and boeravinone J (16, 1.1 mg) (Figure 9). The fraction eluted with \( n \)-hexane-EtOAc 4:6 was purified by HPLC on an analytical column using \( n \)-hexane-EtOAc 4:6 as an eluent, flow rate 0.8 ml/min, yielding boeravinone C (6, 5.1 mg). Finally, the fraction eluted with EtOAc-\( n \)-hexane 7:3 was further purified by HPLC (EtOAc-\( n \)-hexane, 6:4, flow rate 0.8 ml/min,) yielding coccineone B (11, 3.9 mg) (Figure 9).

*Boeravinone I* (15): it is pale yellow amorphous solid; \([\alpha]^{25}\text{D}\) 0 (c0.01, MeOH); UV (CH\(_3\)OH): \(\lambda_{\text{max}}\) (log \(\varepsilon\)) 330 nm (3.85), 274 nm (4.50); UV (CH\(_3\)OH + AlCl\(_3\)): \(\lambda_{\text{max}}\) 363, 280 nm; UV (CH\(_3\)OH + AlCl\(_3\)/HCl): \(\lambda_{\text{max}}\) 371, 283 nm; UV (CH\(_3\)OH + NaOAc/H\(_3\)BO\(_3\)): \(\lambda_{\text{max}}\) 331, 274 nm; 1H NMR (500 MHz, CD\(_3\)OD) and 13C NMR (125 MHz, CD\(_3\)OD): *see Appendix*. ESIMS
341.0666 (calcd for C_{18}H_{13}O_7, m/z 341.0661). (negative-ion) m/z 341 [M - H]-; HRFABMS (negative ions): found m/z

*Boeravinone J (16)*: it is yellow amorphous solid; UV (CH₃OH): λ_{max} (log ε) 340 nm (3.40), 276 nm (4.40); UV (CH₃OH + AlCl₃): λ_{max} 374, 283 nm; UV (CH₃OH + NaOAc/H₃BO₃): λ_{max} 340, 276 nm; 1H NMR (500 MHz, CD₃OD) and 13C NMR (125 MHz, CD₃OD): see Appendix. ESIMS (negative-ion) m/z 297 [M - H]-; HRFABMS (negative ions) found m/z 297.0405 (calcd for C_{16}H_{9}O_6, m/z 297.0399).

### 3.5.3 Cell Culture

Human embrional kidney cells (HEK-293) transfected by wild-type (R482) BCPR were used.

### 3.5.4 BCPR Inhibition

The inhibitory protein activity of boeravinones was assayed by flow cytometry (Ahmed-Belkacem et al. 2005). The HEK-293 human cells were
transfected by wild-type (R482) BCRP and were exposed to 5 µM mitoxantrone for 30 min at 37 °C, either in the absence or the presence of the inhibitor added as a dimethyl sulfoxide solution (0.5% final concentration), washed in PBS, and further incubated for 60 min with the same inhibitor concentration in the mitoxantrone-free medium. The residual intracellular drug fluorescence was monitored with a FACscan flow cytometer (Becton Dickinson, Moutain View, CA). The maximal fluorescence (100%) was the difference between the mean fluorescence of the control cells (transfected with empty vector) and BCRP-transfected ones incubated with the substrate but without the inhibitor. The same maximal fluorescence with the latter cells was obtained in the presence of 5 µM ELACRIDAR, taken as a reference inhibitor of BCRP-mediated drug efflux. The addition of boeravinones did not significantly modify the fluorescence of the control cells and the cells without the drug were taken as an auto fluorescence control.

3.6. Toxicological Studies
3.6.1 Substances and Drugs

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the LDH leakage commercial kit were purchased from Sigma (Milan, Italy). All the reagents for the cellular cultures were supplied by the Microtech Research Products (Naples, Italy). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was dissolved in sterile PBS.

3.6.2 Bioassay-Guided Isolation of the Active Compounds

The isolation procedure is described on page 34.

3.6.3 MTT Assay

The cellular vitality was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, as described by Mosmann (1983), which is based on the ability of mitochondrial dehydrogenases to cut the tetrazolic ring of the MTT and transform the MTT from a yellow water soluble substance into the dark purple insoluble crystals of formazan. The Caco-2 cells were seeded in 96 multiwell plates at a density of $1 \times 10^4$ cells/well to the final
volume of 200 µl DMEM. After the incubation of the cells (24 hrs) with the *B. diffusa* methanolic root extract (30-300 µg/ml) and boeravinone G (0.0001-0.001 µg/ml), the cell culture was then incubated with the MTT solution (0.25 mg/ml) for 1 hr at 37 °C. The supernatant was removed and the formed formazan crystals were dissolved in DMSO (100 µl/well) at room temperature for 10 min. The absorbance was read at a wavelength (λ) of 490 nm in a multiwell plate reader (Bio-Rad, Model 550).

3.6.4 Lactate Dehydrogenase (LDH) Leakage Assay

Lactate dehydrogenase, also called lactic dehydrogenase (LDH) is an enzyme responsible for converting lactic acid into pyruvic acid, an essential step in producing cellular energy. The LDH leakage assay indicates the cell viability and integrity after the damaging exposure. It is a fluorometric assay that measures the release of LDH from the cells with a damaged membrane by the conversion of resazurin into resorufin (Shimma et al., 2003). The Caco-2 cells were seeded in 6-well plates at the density of 3.0 x10^6 per well and led to
the differentiation. The differentiated cells were treated with the methanolic root extract of *B. diffusa* (30-300 µg/ml) and boeravinone G (0.0001-0.001 µg/ml) for 24 hrs. An aliquot of the medium was removed from the culture plates and then analyzed for LDH leakage into the culture media by using a commercial kit. The total LDH activity was determined after the cells were scraped and thoroughly disrupted by Ultra Turax for 30 seconds. The percentage of LDH leakage was then calculated to determine the membrane integrity. The LDH leakage was expressed as a percentage of the total activity:

\[
\frac{\text{activity in the medium}}{\text{activity in the medium} + \text{activity of the cells}} \times 100.
\]

**3.6.5 Genotoxicity Assay (Comet Assay)**

In this assay the presence of the DNA fragmentation was examined by the single cell gel electrophoresis (Comet assay), as described by Gill et al. (2005). The Caco-2 cells were seeded in the 25 cm² flasks at the density of the 4x10^5 cells and incubated with the *B. diffusa* methanolic root extract (30-300 µg/ml)
at 37°C for 24 hrs. After the incubation, the potential *B. diffusa*-induced DNA damage was investigated as reported on the page 44.

3.7 Statistical Analysis

The results are expressed as the arithmetic mean ± S.E. mean [or 95% confidence limits (C.L.) of the IC<sub>50</sub> values]. The comparisons between the two sets of data were made by Student’s t-test for paired data. When the multiple comparisons against a single control were made, ANOVA was used, followed by the Bonferroni’s multiple comparisons test. Analysis of variance (two way) was used to compare different cumulative concentration-effect curves. For the genotoxicity test (Comet assay) the data were statistically analyzed in a quantitative way, using a software of image analysis in a position to calculate parameters like “tail length” and “tail moment”. The average percentage of the DNA tail was calculated on 100 cells for the triplet gel and the average of it is used for the analysis statistics. The arithmetic mean IC<sub>50</sub> values and 95% C.L. were calculated by analyzing the regression lines according to Tallarida and
Murray (1996). $E_{max}$ values were calculated using the Graph Pad Instat program version 4.01. A $p$-value of less than 0.05 was considered significant.
4.0 RESULTS

4.1 Spasmolytic Activity

The methanolic root extract of *B. diffusa* (1-1000 µg/ml) significantly, and in a concentration-dependent manner, inhibited the electrical field stimulation (EFS)-, acetylcholine-, histamine-, and BaCl$_2$- induced contractions of the isolated guinea pig ileum (Figure 10). The inhibitory effect of the methanolic root extract of *B. diffusa* on EFS-, acetylcholine-, histamine-, and BaCl$_2$- induced contractions was similar; in fact, the curves were overlapping and the arithmetic IC$_{50}$ mean values (95% C.L.) for EFS, acetylcholine, histamine and BaCl$_2$ were 182 (138-240) µg/ml, 160 (121-212) µg/ml, 168 (132-213) µg/ml, and 158 (117-215) µg/ml, respectively.

The inhibition produced by the methanolic root extract of *B. diffusa* was reversible, since at the end of the experiment (after washing the tissues), the spasmogens produced the contractions with an amplitude similar to the one obtained at the beginning of the experiment (before the treatment with the
Figure 10. Effect of the methanolic root extract of Boerhaavia diffusa (1-1000 µg/ml) on contractions induced by acetylcholine (10⁻⁶ M), histamine (10⁻⁶ M), barium chloride (10⁻⁴ M), and electrical field stimulation (EFS, 2.5 Hz for 2 s, 400 mA, 1 ms pulse duration) on isolated guinea pig ileum. Each point represents the mean ± SEM of 6-8 experiments.
Since the *B. diffusa* methanolic root extract inhibited the contractions induced by three different spasmogens, a common site of an action on the smooth muscle was hypothesized, for example, the involvement of cytosolic Ca\(^{2+}\), which is known to play an important role in the contractile processes of smooth muscle (Makhlouf, 1994). Therefore, the action of calcium antagonists/blockers on the spasmolytic effect of the *B. diffusa* methanolic root extract was evaluated. The inhibitory response of *B. diffusa* on the ACh-induced contractions was significantly (*p* <0.05) reduced by nifedipine (10\(^{-6}\) M) or EDTA (10\(^{-3}\) M) (Figure 11): IC\(_{50}\) values (95% C.L.) for the *B. diffusa* methanolic root extract in the presence of nifedipine or EDTA were 315 (204-488) µg/ml and 228 (179-290) µg/ml, respectively. In contrast, cyclopiazonic acid (10\(^{-5}\)M) significantly increased the inhibitory response induced by the *B. diffusa* root extract with IC\(_{50}\) value (95% C.L.)of 5.44 (3.95-7.49) µg/ml (Figure 11). The effect of nifedipine, a Ca\(^{2+}\) channel blocker of L-type and
EDTA, a calcium chelator, on the inhibitory effect of the *B. diffusa* methanolic root extract on the ACh-induced contractions suggest an involvement of extracellular calcium and/or L-type calcium channels.

Conversely, cyclopiazonic acid, a potent and specific inhibitor of the sarcoplasmatic reticulum Ca$^{2+}$-ATPase in smooth muscle (i) reduced the inhibitory effect of the methanolic root extract of *B. diffusa* on the ACh-induced contractions and (ii) produced a leftward shift of the inhibitory curve (Figure 11).

The methanolic root extract of *B. diffusa* was then subjected to the modified Kupchan partition scheme from which four fractions were obtained (*n*-hexane, CCl$_4$, CHCl$_3$, and *n*-BuOH fractions). The obtained extracts were subjected to the preliminary evaluation of their effect on intestinal motility. This test revealed the maximum spasmolytic activity for the CCl$_4$ and CHCl$_3$ extracts, while the remaining two fractions were inactive (Figure 12).

In order to get more information on the smooth muscle contraction...
Figure 11. Acetylcholine-induced contractions in isolated guinea pig ileum: effect of the methanolic root extract of *Boerhaavia diffusa* (1-1000 µg/ml) alone or in the presence of nifedipine (10^{-6} M), cyclopiazonic acid (10^{-5} M) and EDTA (10^{-3} M). Each point represents the mean ± SEM of 6-8 experiments. **$p < 0.01$; ***$p < 0.001$ vs vehicle.
Figure 12. Effects of chloroformic (CHCl₃), tetrachloruric (CCl₄), n-hexane and butanolic (BuOH) fractions obtained from the methanolic root extract of *B. diffusa* (1-1000 μg/ml) on contractions on isolated guinea pig ileum induced by acetylcholine (Ach, 10⁻⁶ M). Each point represents the mean ± SEM of 6-8 experiments.
inhibitory activity of the nonprenylated rotenoids contained in the *B. diffusa* root, the methanolic root extract of this plant was carefully examined and the twelve rotenoid derivatives (1-12) were isolated, three of which are new compounds (4, 5 and 12) (Figure 13).

The evaluation of the spasmolytic effect of the twelve isolated rotenoids (1-12) showed that the compounds 2, 3 and 5, but not the compounds 1 and 4, were able to reduce the acetylcholine-induced contractions with the following IC₅₀ value (95% C.L.): 11.0 (10.2 – 12.3) µg/ml for boeravinone E (2), 4.22 (2.16 – 8.21) µg/ml for boeravinone G (5) and 5.46 (4.45 – 6.69) µg/ml for the compound 3. In addition, the compounds 2 and 3, at the concentration of 30 µg/ml, produced a complete inhibition of the acetylcholine-induced contractions, while the maximal inhibition achieved by boeravinone G (5) at the concentration of 30 µg/ml was 60% (Table 1). The compounds 7-10 and 12 reduced the acetylcholine-induced contractions (at 30 µg/ml). By contrast, the compounds 6 and 11 were completely inactive. None of these compounds
Figure 13. Rotenoids isolated from the methanolic root extract of *Boerhaavia diffusa* and tested on intestinal motility.
(6-12) inhibited the acetylcholine-induced contractions completely: indeed, the maximal inhibition ($E_{max}$) achieved for each of the compounds 7-10 and 12, at the tested concentrations, was about 20-50% (Table 1). Papaverine, used as a reference drug, reduced the acetylcholine-induced contractions with a percentage of maximum inhibition of $72.3 \pm 7.6$ at the concentration of 30 µg/ml (Table 1).

4.2 Antioxidant Activity

4.2.1 Electron Spin Resonance Spectroscopy (ESR)

The methanolic root extract of B. diffusa (5 mg/ml) induced a significant peak height reduction of the absorbance (B signal) in relation to the control peak height (A signal) (Figure 14). The variation of the absorption of the peak height presents the measure of the antiradical activity. As shown in Figure 14, the reaction of Fe$^{2+}$ with H$_2$O$_2$ in the presence of the spin trapping agent DMPO, generated a 1:2:2:1 quartet of lines in the ESR spectra with the hyperfine coupling parameters ($a_N$ and $a_H=14.9$ G).
Table 1. Inhibition of ACh-induced contractions by rotenoids 1-12 at 30 µg/ml.

<table>
<thead>
<tr>
<th>Rotenoids</th>
<th>$E_{max}^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>boeravinone D (1)</td>
<td>Inactive$^b$</td>
</tr>
<tr>
<td>boeravinone E (2)</td>
<td>100</td>
</tr>
<tr>
<td>compound 3</td>
<td>100</td>
</tr>
<tr>
<td>boeravinone G (4)</td>
<td>60.0±6.9</td>
</tr>
<tr>
<td>boeravinone H (5)</td>
<td>Inactive$^b$</td>
</tr>
<tr>
<td>boeravinone C (6)</td>
<td>Inactive</td>
</tr>
<tr>
<td>compound 7</td>
<td>19.4 ± 1.3</td>
</tr>
<tr>
<td>coccineone E (8)</td>
<td>25.2 ± 2.0</td>
</tr>
<tr>
<td>isoflavone 9</td>
<td>36.8 ± 3.2</td>
</tr>
<tr>
<td>boeravinone F (10)</td>
<td>50.0 ± 8.2</td>
</tr>
<tr>
<td>coccineone B (11)</td>
<td>Inactive$^b$</td>
</tr>
<tr>
<td>compound 12</td>
<td>36.1 ± 3.3</td>
</tr>
<tr>
<td>papaverine</td>
<td>72.3±7.6</td>
</tr>
</tbody>
</table>

$^a$ $E_{max}$ indicates the percentage of maximum inhibition.

$^b$ Inactive means with no effect on ACh-induced contractions.
Figure 14. Representative ESR spectra of DMPO-OH spin adduct signal (A) and DMPO-OH spin adduct signal in the presence of 5 mg/ml of *Boerhaavia diffusa* methanolic root extract (B).
The methanolic root extract of *B. diffusa* (0.1-5 mg/ml) produced a concentration-dependent inhibition of the ESR signal intensity of the DMPO-OH spin adduct with the IC$_{50}$ of 0.7 mg/ml (Table 2).

The CCl$_4$ and the $n$-BuOH fractions, among the four ones (*n*-hexane, CCl$_4$, CHCl$_3$, $n$-BuOH) obtained by a modified Kupchan procedure, were able to reduce the ESR signal intensity, where the former was more active (Table 3). The carbon tetrachloride extract produced a total elimination of hydroxyl radical (AA = 100%) at the concentration of 0.7 mg/ml.

The chromatographic purification of the CCl$_4$ fraction through column chromatography on a silica gel led to the formation of 13 sub-fractions whose antioxidant activity was evaluated by ESR assay (Table 4).

The HPLC purification of the most active sub-fractions (BOE 4, BOE 6, BOE 8 and BOE 9) led to the isolation of boeravinone G, boeravinone H and boeravinone D (structures in Figure 13) which, at the concentration of 0.5 mg/ml, showed a scavenger activity of 65.9±3.3 %, 50.2±2.4 % and
Table 2: Antioxidant activity of the methanolic root extract of *Boerhaavia diffusa* (0.1-5 mg/ml) detected by ESR assay, expressed as percentage of the reduction of peak height of the absorbance. Each value represents the mean ± SEM of 6 experiments. **p<0.01 e ***p<0.001.

<table>
<thead>
<tr>
<th><em>Boerhaavia diffusa</em> (mg/ml)</th>
<th>%AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>12.2 ± 0.51</td>
</tr>
<tr>
<td>0.5</td>
<td>26.55 ± 0.62</td>
</tr>
<tr>
<td>1.0</td>
<td>52.32 ± 0.98**</td>
</tr>
<tr>
<td>3.0</td>
<td>66.9 ± 0.57***</td>
</tr>
<tr>
<td>5.0</td>
<td>71.22 ± 0.43***</td>
</tr>
</tbody>
</table>
**Table 3:** Antioxidant activity, detected by ESR assay, of the fractions obtained from Kupchan partitioning of the methanolic root extract of *Boerhaavia diffusa* expressed as percentage of peak height of the absorbance.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>0.1 mg/ml</th>
<th>0.5 mg/ml</th>
<th>1 mg/ml</th>
<th>3 mg/ml</th>
<th>5 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cloroformic</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tetacloruric</td>
<td>59.9±0.87</td>
<td>85.5±0.84</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Butanolic</td>
<td>15.5±0.54</td>
<td>35.6±0.73</td>
<td>61.2±0.89</td>
<td>68.9±0.91</td>
<td>78.1±0.41</td>
</tr>
</tbody>
</table>
Table 4: Antioxidant activity of the fractions obtained from the carbon tetrachloride extract of *Boerhaavia diffusa* root detected by ESR assay

<table>
<thead>
<tr>
<th>Sub fractions</th>
<th>0.01 mg/ml</th>
<th>0.15 mg/ml</th>
<th>1.00 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOE 1</td>
<td>26.32</td>
<td>76.87</td>
<td>91.50</td>
</tr>
<tr>
<td>BOE 2</td>
<td>24.41</td>
<td>78.05</td>
<td>92.20</td>
</tr>
<tr>
<td>BOE 3</td>
<td>23.22</td>
<td>77.45</td>
<td>88.45</td>
</tr>
<tr>
<td>BOE 4</td>
<td>31.24</td>
<td>82.93</td>
<td>98.75</td>
</tr>
<tr>
<td>BOE 5</td>
<td>20.13</td>
<td>65.85</td>
<td>82.31</td>
</tr>
<tr>
<td>BOE 6</td>
<td>30.12</td>
<td>81.71</td>
<td>100</td>
</tr>
<tr>
<td>BOE 7</td>
<td>24.13</td>
<td>75.61</td>
<td>86.71</td>
</tr>
<tr>
<td>BOE 8</td>
<td>33.34</td>
<td>82.32</td>
<td>100</td>
</tr>
<tr>
<td>BOE 9</td>
<td>32.98</td>
<td>81.71</td>
<td>100</td>
</tr>
<tr>
<td>BOE 10</td>
<td>0</td>
<td>12.19</td>
<td>51.25</td>
</tr>
<tr>
<td>BOE 11</td>
<td>22.32</td>
<td>75.61</td>
<td>90.81</td>
</tr>
<tr>
<td>BOE 12</td>
<td>15.12</td>
<td>51.22</td>
<td>75.25</td>
</tr>
<tr>
<td>BOE 13</td>
<td>20.12</td>
<td>75.61</td>
<td>88.45</td>
</tr>
</tbody>
</table>
Boeravinone G (1,0 mg/ml), isolated from the BOE 4 fraction, showed the antiradical activity (AA%) (expressed as a percentage of the peak height of the absorbance) of the 95.9±4.6 %.

### 4.2.2 Sucrase-Isomaltase Activity

In order to evaluate the differentiation grade of the Caco-2 cells a time course of the sucrase-isomaltase enzymatic activity was performed. The sucrase-isomaltase activity increased in a time-dependent manner until the day 12 (Figure 15).

### 4.2.3 TBARS Assay

Lipid peroxidation is considered as the major mechanism of cellular injury in many biological systems of plant and animal origin. The mechanism involves the process whereby the unsaturated lipids are oxidized to form the additional radical species as well as toxic by-products that can be harmful to the host system. In this study, the lipid peroxidation was determined by
Figure 15. Time course of sucrase-isomaltase activity. Each value represents the mean ± SEM of 4 experiments.
measuring TBARS. The treatment of the Caco-2 cells with H$_2$O$_2$/Fe$^{2+}$ (1 mM) produced a significant (p<0.001) threefold increase in TBARS formation (Figures 16 and 17). The pre-treatment of the Caco-2 cells with the methanolic root extract of *B. diffusa* (30-300 µg/ml) reduced the H$_2$O$_2$/Fe$^{2+}$-induced TBARS formation significantly (p<0.01-0.001) in a concentration-related manner (Figure 16). The effect was significant starting from the concentration of 30 µg/ml. Similarly, boeravinone G (0.0001-0.001 µg/ml) was able to reduce the levels of TBARS significantly (p<0.001) and in a concentration dependent manner (Figure 17).

**4.2.4 Intracellular ROS Measurement**

The oxidative stress is a condition associated with an increased rate of the cellular damage induced by oxygen and oxygen-derived oxidants called reactive oxygen species (ROS). The exposure of the Caco-2 cells to H$_2$O$_2$/Fe$^{2+}$ (2mM) produced a significant (p<0.05-0.001) increase in the ROS formation (Figures 18 and 19). The pre-treatment for 24 hrs with the methanolic
Figure 16. Effect of the methanolic root extract of *Boerhaavia diffusa* (30-300 µg/ml) on Fenton reagent (H₂O₂/Fe²⁺ 1 mM)-induced malondialdehyde (MDA) production after 24 hrs of exposure in differentiated Caco-2 cells. Data represent mean ± S.E.M of 6 experiments. #p<0.001 vs control; **p<0.01 and ***p<0.001 vs H₂O₂/Fe²⁺ alone.
Figure 17. Effect of boeravinone G (0.0001-0.001 μg/ml) on Fenton reagent (H₂O₂/Fe²⁺ 1mM)-induced malondialdehyde (MDA) production after 24 hrs of exposure in differentiated Caco-2 cells. Data represent mean ± S.E.M of 6 experiments. #p<0.001 vs control; ***p<0.001 vs H₂O₂/Fe⁺² alone.
root extract of *B. diffusa* (30-300 µg/ml) or boeravinone G (0.0001-0.001 µg/ml) reduced significantly (p<0.05-0.001) and in a concentration dependent manner the ROS formation as measured by the inhibition of DCF fluorescence intensity (Figures 18 and 19).

### 4.3 Genoprotective Activity (Comet Assay)

The treatment of the Caco-2 cells with H₂O₂ 75 µM induced the double helix DNA breakage of the Caco-2 cells. The DNA fluorescence tail of the H₂O₂-damaged Caco-2 cells was about 43%, while the control was about 5%. A pre-treatment with the methanolic root extract of *B. diffusa* (30-300 µg/ml) reduced the DNA damage significantly (p<0.01-0.001) and in a concentration dependent manner (Figure 20). Consistent with the TBARS assay, a significant inhibitory effect was achieved for the 30-300 µg/ml concentrations. Figure 21 shows an example of the Caco-2 cells exposed to 75 µg/ml H₂O₂ or H₂O₂ plus the methanolic root extract of *B. diffusa*. 
**Figure 18.** Effect of the methanolic root extract of *Boerhaavia diffusa* (30-300 µg/ml) on Fenton reagent (H$_2$O$_2$/Fe$^{2+}$ 2 mM)-induced reactive species reagents (ROS) production after 24-hrs exposure in differentiated Caco-2 cells. Data represent mean ± S.E.M of 6 experiments. #p<0.001 vs control; *p<0.05 and ***p<0.001 vs H$_2$O$_2$/Fe$^{2+}$ alone.
Figure 19. Effect of boeravinone G (0.0001-0.001 µg/ml) on Fenton reagent (H$_2$O$_2$/Fe$^{2+}$ 2 mM)-induced reactive species reagents (ROS) production after 24-hrs exposure in differentiated Caco-2 cells. Data represent mean ± S.E.M of 6 experiments. 

#p<0.01 vs control; *p<0.05, **p<0.01 and ***p<0.001 vs H$_2$O$_2$/Fe$^{2+}$ alone.
Figure 20. DNA damage (tail intensity) detected by the Comet assay in Caco-2 cell line exposed to 75 µM H₂O₂ for 5 min. in absence or presence of the methanolic root extract of *Boerhaavia diffusa* (30-300 µg/ml). Data represent mean ± S.E.M of 4 experiments. #p<0.001 vs control; **p<0.01 and ***p<0.001 vs H₂O₂ alone.
Figure 21. Examples of Comet images showing different levels of DNA damage in Caco-2 cells.

(a) Comet showing 50% of the DNA damage induced by H₂O₂ 75 µM
(b) Comet showing 30% of the DNA damage induced by H₂O₂ 75 µM after 24-hrs pre-treatment with the methanol root extract of Boerhaavia diffusa (300 µg/ml)
4.4 BCRP Mediated Inhibition Assay

The rotenoids isolated from *B. diffusa* were assayed for the inhibition of the BCRP activity by flow cytometry on HEK-293 human cells transfected by wild-type (R482) BCRP and exposed to the antitumor drug mitoxantrone (Ahmed-Belkacem et al., 2005). Table 5 shows the efficiency of boeravinone G, boeravinone H, boeravinone E, boeravinone B, boeravinone C, coccineone B, boeravinone A, coccineone E, 6-Ο-dimethylboeravinone H, boeravinone I and boeravinone J to inhibit BCRP-mediated mitoxantrone efflux, leading to the drug accumulation (Figure 9). Boeravinone G and boeravinone H induced a high accumulation of mitoxantrone at the lowest concentration tested, through a strong inhibition of the BCRP drug-efflux activity (Figure 22). For boeravinone G, nearly the complete effect was achieved that is produced by elacridar (GF120918), a commonly used BCRP inhibitor, taken here as a reference substance. A concentration dependent study enabled the determination of IC\textsubscript{50} values of 0.7 ± 0.07 μM for boeravinone G and 2.5 ±
Figure 22. Concentration-dependent inhibition by boeravinone G and boeravinone H of BCRP-mediated mitoxantrone efflux. BCRP-transfected HEK-293 cells were incubated as in Table 5 in the presence of increasing concentrations of either boeravinone G (panel A) or boeravinone H (panel B). The data obtained from three separate experiments are indicated as the mean ± SD. The IC₅₀ was determined graphically as the concentration producing half-maximal mitoxantrone accumulation.
0.47 μM for boeravinone H, respectively. Most of the remaining boeravinones (boeravinone A, boeravinone B, boeravinone E, coccineone B and boeravinone C) were less active and required the higher concentrations to produce appreciable inhibition, while 6-\textit{O}-dimethylboeravinone H and the new boeravinone I and boeravinone J displayed only a marginal inhibitory activity (Table 5).

4. 5 Toxicological Studies

4.5.1 Cytotoxicity Assay

4.5.1.1 MTT Assay

The results regarding the citotoxicity assay on the methanolic root extract of \textit{B. diffusa} and on boeravinone G, performed on the Caco-2 cells are shown on the Figure 23 and Figure 24. The exposure of the differentiated Caco-2 cells to the various concentrations of the methanolic root extract of \textit{B. diffusa} 1-300 μg/ml (Figure 23) and to the most antioxidant rotenoid boeravinone G
Table 5. Efficacy of rotenoids to inhibit BCRP-mediated mitoxantrone efflux leading to the drug accumulation

<table>
<thead>
<tr>
<th>Sostituents</th>
<th>Concentration used (µM)</th>
<th>% maximal mitoxantrone accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>boeravinone G</td>
<td>H OH OMe H OMe H DB</td>
<td>92 ± 6.5</td>
</tr>
<tr>
<td>boeravinone H</td>
<td>H OH OMe H OMe Me DB</td>
<td>68 ± 6.1</td>
</tr>
<tr>
<td>boeravinone E</td>
<td>OH H OH H OH Me DB</td>
<td>56 ± 5.0</td>
</tr>
<tr>
<td>boeravinone B</td>
<td>H H OH H OH Me DB</td>
<td>55 ± 5.8</td>
</tr>
<tr>
<td>boeravinone C</td>
<td>H OH H H OMe Me 12a-OH</td>
<td>31 ± 4.2</td>
</tr>
<tr>
<td>coccineone B</td>
<td>H H OH H OH H Db</td>
<td>29 ± 5.3</td>
</tr>
<tr>
<td>boeravinone A</td>
<td>H H OMe H OH Me Db</td>
<td>27 ± 5.1</td>
</tr>
<tr>
<td>coccineone E</td>
<td>H H H H OMe OMe 12a-OH</td>
<td>15 ± 5.2</td>
</tr>
<tr>
<td>6-O-dimethylboeravinone H</td>
<td>H OH OH H OMe Me DB</td>
<td>15 ± 3.1</td>
</tr>
<tr>
<td>boeravinone I</td>
<td>H H OH OH OMe Me DB</td>
<td>12 ± 5.4</td>
</tr>
<tr>
<td>boeravinone J</td>
<td>OH H B ring with 5 atoms H OH Me DB</td>
<td>15 ± 3.1</td>
</tr>
</tbody>
</table>
Figure 23. Effect of the methanolic root extract of *Boerhaavia diffusa* (1-300 µg/ml) on the differentiated the Caco-2 cells. Data represent mean ± S.E.M of 8 experiments.
Figure 24. The exposure of the differentiated Caco-2 cells to the various concentrations of the most antioxidant rotenoid boeravinone G (0.0001-0.001 µg/ml). Data represent mean ± S.E.M of 6 experiments.
(0.0001-0.001 µg/ml corresponding to 0.28-2.8 µM) (Figure 24) resulted in no

effect on the cell survival.

4.4.1.2 Lactate Dehydrogenase (LDH) Leakage Assay

In accordance with the results of the MTT assay, the methanol root extract

of Boerhaavia diffusa (1-300 µg/ml) (Figure 25) and boeravinone G (0.0001-

0.001 µg/ml) (Figure 26) did not produce an increase in the release of LDH

from the Caco-2 cell line.

4.4.2 Genotoxicity Assay (Comet Assay)

The Comet assay is used not only to determinate the capacity of a drug to

protect the DNA from insults, but it is also used to evaluate the genotoxicity of

a drug. In our study, the methanolic root extract of B. diffusa, at the

concentrations ranging from 30 to 300 µg/ml, did not produce the DNA

damage detected by the Comet assay in the Caco-2 cells. Thus, this result

excluded a genotoxic activity of the B. diffusa methanolic root extract (Figure

27).
Figure 25. Exposure of the differentiated Caco-2 cells to the various concentrations of the methanolic root extract of *Boerhaavia diffusa* (1-300 µg/ml). Data represent mean ± S.E.M of 6 experiments.
Figure 26. Exposure of the differentiated Caco-2 cells to the various concentrations of the most antioxidant rotenoid boeravinone G (0.0001-0.001 µg/ml). Data represent mean ± S.E.M of 6 experiments.
Figure 27. DNA damage (tail intensity) detected by the Comet assay in Caco-2 cell line exposed to the methanolic root extract of *Boerhaavia diffusa* (30-300 µg/ml). Data represent mean ± S.E.M of 4 experiments.
5.0 DISCUSSION

*Boerhaavia diffusa* is one of the most popular herbal remedies in the Indian Ayurvedic medicine that prescribes roots of *Punarnava* as diuretic, expectorant, vermifuge, analgesic and to improve the functions of kidney and liver (Lad, 1999). *B. diffusa* is also widely used in Ayurvedic medicine for the treatment of various gastro-intestinal disorders such as diarrhoea and abdominal pain. However, despite its wide use, there were not carried out experimental and clinical studies that confirm its effectiveness.

*Spasmolytic Activity*

Gastrointestinal diseases are acute and severe public health problems all over the world. It has been assessed, for example, that 1.4 million people in the United States and 2.2 million people in Europe suffer from diarrhoea, constipation or abdominal pain (Loftus, 2004).

In the present study, we tried to investigate the effect of a methanolic root extract of *B. diffusa* on intestinal motility. In particular, we evaluated the effect
of *B. diffusa* root extract on the contractions induced either by exogenous spasmogens (acetylcholine, histamine or barium chloride) or by electrical field stimulation (EFS) of the isolated guinea-pig ileum.

Our results showed that a methanolic extract obtained from the root of *B. diffusa* exhibited a concentration-dependent inhibition of both exogenous spasmogens (i.e., acetylcholine, histamine, and barium chloride) and EFS-evoked contractions in the isolated guinea pig ileum. Acetylcholine, histamine, BaCl₂ and EFS induced the ileum contraction by different mechanisms: acetylcholine and histamine act through the activation of postjunctional receptors while EFS acts through the release of acetylcholine from enteric nerves; BaCl₂ through a postjunctional non-receptor-mediated mechanism. Since no significant difference was observed in the *B. diffusa* inhibition curves, the spasmolytic effect of *B. diffusa* is probably due to a direct effect on smooth muscle. It is very unlikely that the antispasmodic effect of the methanolic root extract of *B. diffusa* is due to antimuscarinic action (atropine-like), since this
herbal drug also inhibited the contractions induced either by BaCl₂ (which does not act through a receptor-mediated mechanism) or histamine (which acts on H₁ receptors).

Since the inhibitory effect of the methanolic root extract of *B. diffusa* was exerted at post-junctional level, we investigated the mechanism of the antispasmodic activity of this herbal extract on the contractions induced by acetylcholine.

The contractions of all smooth muscles, including those of gastrointestinal tract, absolutely depend on the presence of calcium (Ca²⁺). Agonists-induced contractions may be related to the release of intracellular Ca²⁺ from the sarcoplasmic stores in addition to the influx of extracellular Ca²⁺, mainly through the L-type Ca²⁺ channels (Makhlouf, 1994). Consequently, smooth muscle contraction can be abolished by antispasmodic drugs through the inhibition of Ca²⁺ entry or release into the cells. In the present study, we have shown that nifedipine, a blocker of the L-type Ca²⁺ channels and EDTA, a
calcium chelator, reduced the inhibitory effect of the methanolic root extract of
*B. diffusa* on acetylcholine-induced contractions suggesting an involvement of
L-type channels. Cyclopiazonic acid, a potent and specific inhibitor of the
sarcoplasmic reticulum Ca\(^{2+}\)-ATPase in smooth muscle (Grasa et al., 2004),
did not reduce the inhibitory effect of the methanolic root extract of *B. diffusa*
on ACh-induced contractions, but instead produced a leftward shift of the
inhibitory curve. This action excludes an involvement of the release of Ca\(^{2+}\)
from sarcoplasmic stores in the inhibitory action of the methanolic root extract
of *B. diffusa*.

In order to distinguish the responsible metabolites of the spasmolytic
activity of *B. diffusa* methanol root extract, the extract was fractioned following
the modified Kupchan method (Kupchan et al., 1973) in five fractions: hexane,
tetrachloruric, chloroformic, butanolic and aqueous fractions. These fractions
were subjected to preliminary evaluation of their effect on intestinal motility.
This test revealed a maximum spasmolytic activity for CCl\(_4\) and CHCl\(_3\)
fractions, while the remaining three fractions were inactive. Through a bioassay-guided separation, twelve compounds belonging to the rotenoids class (Figure 7) were isolated, three of them isolated for the first time and named boeravinone G (5), boeravinone H (4) and 6,10,11-trihydroxy-9-methoxyrotenone (12).

Pharmacological tests showed that only boeravinone E (2, 100 % of inhibition), boeravinone G (5, 60 % of inhibition) and 6-\textit{O}-demethylboeravinone H (3, 100 % of inhibition) exhibited a high spasmolytic activity, the compounds 7-10 and 12 reduced acetylcholine-induced contractions (at 30 µg/ml) at about 30%, while, in contrast, boeravinone H (4), boeravinone C (6) and coccineone B (11) were completely inactive.

The evaluation of the spasmolytic activity within the series of rotenoids isolated from the methanolic root extract of \textit{B. diffusa} during the present study allowed to get a complete formulation of structure-activity-relationship, in particular to estimate the effect of substitutions at the two aromatic rings and of
O-methylation at C-6 on the spasmolytic activity. In particular, the comparison of the activities of boeravinone D (1) with boeravinone E (2) and those of boeravinone H (4) with 6-O-demethylboeravinone H (3) clearly suggested that O-methylation at position 6 was deleterious for the pharmacological activity (at 30 µg/ml, only boeravinone E (2) and 6-O-demethylboeravinone H (3) produced a complete inhibition of ACh-induced contractions while the maximal inhibition achieved by boeravinone G (5) at the concentration of 30 µg/ml was 60 %. On the other hand, the same methylation seemed to be better tolerated when a methyl was lacking on the ring D (it is the case of boeravinone G (5)), which retained a good activity. Finally, comparison of the activities of boeravinone E (2) and 6-O-demethylboeravinone H (3) suggested that the linkage position of the OH group on ring A was not critical.

The activities of compounds 6-10 clearly highlighted the crucial role of the ring B (see Table 1). Indeed, the compounds 6-8, showing hydration of the double bond, Δ^6α(12α), irregardless of the presence of either a H, a CH₃, or an
OCH₃ at C-10, showed a reduced activity or no activity. By analogy, the isoflavone 9, characterized by an opened ring B, possessed a low activity. On the other hand, the oxidation of the hemiacetal group at C-6 to a lactone group (as in compound 10) appeared to be better tolerated; however, the activity of boeravinone F (10) was markedly lower compared to its reduced analogue, boeravinone E (2), which appeared to be the most potent rotenoid tested. Finally, the low potencies of the compounds 11 and 12 indicated that the presence of a hydroxylated pyran ring B is not sufficient for the exhibition of spasmolytic activity. Most likely, a monohydroxylated ring A and/or a trisubstituted ring D are also needed.

The data presented herein strengthen the observation that non-prenylated rotenoids are responsible for the smooth muscle relaxant activity of the investigated B. diffusa methanolic root extract. It should be noted that an action on intestinal motility has not been reported previously for this class of molecule. On the other hand, the structurally comparable stilbenoids to some
extent from *Nidema boothii*, have been reported recently to show a spasmolytic activity (Hernandez-Romero et al., 2004). Interestingly, structure-activity relationships established for stilbenoids (hydroxylation of both phenyl rings is needed, while methylation of hydroxyl groups is detrimental) resemble those proposed herein for non-prenylated rotenoids.

In conclusion, the present results demonstrate that the methanolic root extract of *B. diffusa* directly inhibits the intestinal motility with a mechanism involving the L-type Ca\(^{2+}\) channels. Moreover, this action seems due to the presence of rotenoids. These results could explain, at least in part, the clinical efficacy of this Ayurvedic remedy in the treatment of gastrointestinal disorders.

*Antioxidant Activity*

There is a growing evidence that oxygen-derived free radicals play an important role in the pathogenesis of the gastrointestinal disorders. Free radicals are highly reactive species that can induce molecular alterations that involve changes in the morphology and cell viability: these changes include
damage of DNA and the oxidation of protein cross-bonds and its lateral chains.

Free radicals (reactive oxygen species, ROS) are products of "wastes" that are formed naturally in the cells when oxygen is used in metabolic processes to produce energy (oxidation). The lipid peroxidation, for example, is one of the main factors responsible for the functional and structural alterations of the cell membrane caused by oxidative stress (Ames et al., 1993; Cotelle et al., 1996; Halliwell et al., 2000; Brand-Williams et al., 2001). The cells are naturally provided with a large number of protective antioxidant systems, enzyme and non-enzymatic (such as superoxide dismutase-SOD, catalase, etc.) contrasting these potentially harmful oxidizing agents. This multifunctional protective system, however, does not entirely counteract the deleterious effects of ROS and consequently damaged molecules from oxidative phenomena accumulate in cells. The clinical implications of these changes can be serious, in fact, the accumulation of ROS in many cellular components is known to be the main cause of damage of molecules that leads to cell aging and related diseases, such
as cancer and decline of the immune system (Milić et al., 2000). One of the systems to prevent cellular damage mediated by the ROS is to increase the body defences through exogenous antioxidants (Fahey et al., 1997). Consistently, antioxidant compounds are believed to prevent a number of gastrointestinal diseases (Janne et al., 2000; Gatof et al., 2002; Piquet et al., 2006) and drugs with antioxidant activity are currently used in the treatment of gastrointestinal cancer (Trueba et al., 2004; Oz et al., 2005; Dryden et al., 2006). Since *B. diffusa*, is a medicinal plant widely used in Ayurvedic medicine for the treatment gastrointestinal disorders, in the present thesis we evaluated if this medicinal plant possesses antioxidant properties.

One of the approaches to assess the antioxidant activity is to examine free radical production directly and its inhibition by using highly sensitive Electron Spin Resonance (ESR) spectroscopy. The ESR is an analytical assay (discovered and applied for the first time by Zavoiskj in 1944) that measures the presence, structure, dynamics and concentration of free radicals directly
(Calliste et al., 2001). Applying this method it is possible to evaluate the effect of the drug on the formation of radical species obtained by the Fenton reaction which provides for the use of hydrogen peroxide (H₂O₂) and ferrous sulphate (FeSO₄) as oxidizing agents.

Since the hydroxyl radicals are very unstable, it is necessary to use an exogenous spin trap [5,5-dimethyl-1-pyrroline-N-oxide (DMPO)] that reacts with the free radical species thus generating more stable adducts (increasing their “half life”) with the characteristic ESR profiles. Using the ESR assay in the present thesis, it has been found that the methanolic root extract of B. diffusa (reducing the signal intensity of ESR) possesses a scavenging activity.

Using the Caco-2 cell line and H₂O₂ as a free radical generator, the antioxidant effect of the methanolic root extract of B. diffusa was further investigated by evaluating the MDA and the ROS production.

Lipid peroxidation is a complex process that can occur in biological membranes that are made up of molecular oxygen-reactant polyunsaturated
fatty acids, leading to the production of lipid hydroperoxides and their metabolites. The lipid peroxidation can occur in various pathological conditions including atherosclerosis, rheumatoid arthritis, angina, cancer and irritable bowel diseases (IBD).

A simple and largely used method involves thiobarbituric acid-reactive substances which are highly used as lipid peroxidation markers (e.g. like malondialdehyde) (Janero, 1990). Hence, malondialdehyde (MDA) is a quite adequate indicator of lipid peroxidation caused by free radicals. Using this assay, various Authors have discovered the antioxidant activity of the *B. diffusa* extract obtained from a different part of this Ayurvedic plant (i.e. from the leaves and not from the root) in liver and kidney of alloxan-induced diabetic rats (Satheesh et al., 2004). In the present thesis, not only was it reported for the first time the antioxidant activity of the methanolic root extract of *B. diffusa* in the intestinal cells using the TBARS assay, but the antioxidant activity is also confirmed using a more specific assay, namely Reactive Oxygen Species
(ROS) formation assay induced by H$_2$O$_2$ using a fluorescent approach. Indeed, although sensitive, the MDA assay is not specific since, many other biological species can react with TBA (Esterbauer and Cheeseman, 1990). In fact, ROS are essential intermediates in oxidative metabolism. Nonetheless, when generated in excess, ROS can damage cells by peroxidizing lipids and disrupting structural proteins, enzymes and nucleic acids. Excess of ROS is generated during various cell stresses, including ischemia/reperfusion, exposure to ionizing and ultraviolet radiation and/or inflammation.

A great number of in vitro experiments have showed that ROS damage DNA, which appears to represent the major target involved in mutagenesis, carcinogenesis and aging cell responses (Nakabeppu et al., 2006; Karihtala and Soini, 2007). The deleterious effects of ROS are counteracted by several mechanisms permitting an efficient antioxidant defence. The antioxidant capacity neutralizes the ROS production under the physiological conditions, but when an excess of ROS is generated (ETC dysfunction, ischemia/reperfusion,
apoptotic process, etc.) or if the antioxidant defence system is afflicted (malnutrition, genetic disease, neurodegenerative disease, etc.), an oxidative stress is created corresponding to the detrimental effect of ROS. Therefore, it has also been decided to evaluate the potential protective effect of the methanol root extract of *B. diffusa* on the ROS-induced DNA damage. The DNA damage, induced by using H\textsubscript{2}O\textsubscript{2}, which is a well-known genotoxic agent able to induce the oxidative DNA damage, was evaluated by the Comet assay, which is a very sensitive method widely used to evaluate the genotoxic/genoprotective effects of drugs. The damage is represented by an increase of the DNA fragments (generated by DNA double strand breaks, single strand breaks and/or strand breaks induced) that have migrated out of the cell nucleus in the form of a characteristic streak similar to the tail of a comet. The length and fragment content of the tail is directly proportional to the amount of DNA damage (Loft et al., 1996; Stenkellner et al., 2001; Verhagen et al., 1997; Verhoenven et al., 1997; Collins, 2004). Our experiments suggest that the protective action of the
methanolic root extract of *B. diffusa*, assessed by TBARS and ROS assays (see above), is closely related to reduction of the DNA damage induced by H$_2$O$_2$.

Indeed, the methanolic root extract of *B. diffusa* was able to reduce H$_2$O$_2$-induced DNA damage significantly at the concentration of 30-300 µg/ml.

In order to detect the chemical components of the methanolic root extract of *B. diffusa* responsible for the antioxidant activity, this extract was partitioned to obtain four extracts (namely *n*-hexane, CCl$_4$, CHCl$_3$, *n*-BuOH). An ESR-guided fractionation of the most antioxidant extract (CCl$_4$) led to the isolation of three rotenoids, boeravinone D, G and H with a remarkable radical scavenging activity. Obtained results identified rotenoids as the active antioxidant components of the methanolic root extract of *B. diffusa*.

![Figure 28. Boeravinone G, boeravinone H and boeravinone D](image-url)
Since our previous investigations revealed that the rotenoid mixture of *B. diffusa* was made up of at least sixteen molecules (Ahmed-Belkacem, et al., 2005; Borrelli et al., 2005), it was surprising to notice that only boeravinones D, G, and H seemed to play a major role in the antioxidant activity of the extract. The chemical structures of these compounds are reported in Figure 28. The common features of boeravinone G, boeravinone D and boeravinone H (Figure 28) are a planar ring C, the presence of free hydroxyl groups on the ring A at the position 11 and the presence of a methoxy group at the position 6. Remarkably, in the pool of rotenoids present in methanolic root extract *B. diffusa* (Ahmed-Belkacem, et al., 2005; Borrelli et al., 2005), boeravinones D, G, and H are the only ones that possess, at the same time, all these features.

From the results of this study, it is clear that the methanolic root extract of *B. diffusa* possesses an antioxidant activity. This effect could be attributed to a donation of hydrogen, to an electron reduction and / or a direct scavenger activities.
Since boeravinone G exhibited a higher activity compared to boeravinone D and H, further experiments in this study were performed on this metabolite. The Caco-2 based assays (MDA and ROS formations) confirmed the antioxidant activity of boeravinone G, and its likely major role in the antioxidant activity of the whole extract.

**BCPR Induced Inhibition**

Breast cancer resistant proteins (BCRP/ABCG2) has been a recently discovered multidrug transporter of cancer cells, belonging to the ABC ("ATP-Binding Cassette") superfamily of membrane proteins (Doyle, et al., 1998; Allikmets, et al., 1998; Miyake et al., 1999; Klein et al., 1999). It is overexpressed in many types of tumors, as acute myeloid leukaemia, even before any chemotherapeutic treatment (Diestra et al., 2002). BCRP is able to efflux a wide spectrum of antitumor drugs, including mitoxantrone, camptothecin derivatives, methothrexate and anthracyclines, similarly to the first discovered and well characterized transporter P-glycoprotein (P-gp).
BCRPs as well as P-gp are largely expressed in human kidney and liver (Hilgendorf et al., 2007). Considering the importance of several Ayurvedic herbal remedies in the treatment of hepatic and kidney deseases, as well as the relevance of *B. diffusa* itself in the adjuvant treatment, the effect of active rotenoids on the BCRP-inhibiting activity was also investigated in our experiments.

Boeravinones G and boeravinone H were resulted as the highly potent inhibitors of BCRP-mediated mitoxantrone efflux in the transfected HEK-293 human cells.

It was possible to establish structure-activity relationships toward the inhibition of BCRP-mediated drug transport activity, identifying the following substituent positive effects: (i) the absence of a methyl substituent at the position 10 in boeravinone G as compared to boeravinone H; (ii) the preference for a methoxy group over a hydroxyl group at the position 6 (boeravinone H versus 6-O-dimethylboeravinone H). It should be noted that when the position...
4 was unsubstituted and a hydroxyl replaced the methoxy group at both positions 9 and 6, the two previous mentioned effects, (i) and (ii), appeared to be reversed (boeravinone B versus coccineone B and boeravinone B versus boeravinone A, respectively); (iii) an intact ring B, since its contraction to a five-membered ring giving a coumaronochromone derivative was clearly detrimental (boeravinone E versus boeravinone J). In contrast, a hydroxyl group at the position 3 appeared to be neutral (boeravinone E versus boeravinone B). Although the compounds boeravinone C and coccineone E had no direct counterpart, it seemed that loss of the double bond $\Delta^{6\alpha(12\alpha)}$ between the rings B and C, and consequently, of the molecular planarity was deleterious for activity. Interestingly, a similar effect, at the same position, for flavanones/flavones was found (Corea et al., 2003). Analogically, the tetrasubstitution of the ring D, present in the compound boeravinone I, appears to be responsible for a marked decrease in activity.

Toxicological Studies
Boeravinones belong to the chemical class of rotenoids, widely used as botanical insecticides and generally characterized by high toxicity (Gutman et al., 1970). Rotenoids are isoflavonoid derivatives typical secondary metabolites of *Leguminosae* and *Fabaceae* plants and, compared to tricyclic isoflavonoids, their peculiar structural features are: i) an additional oxygenated ring (ring B) almost invariable, ii) a 2,3-disubstitution on the ring A and iii) a prenyl group isoprenoid-derived group attached to the ring D usually at the position 8, commonly cyclized to form a five- or a six-membered ring (ring E) (Figure 29).

![Figure 29](image)

**Figure 29.** Left: General structure of rotenoids from *Leguminosae* and *Fabaceae*. Right: General structure of rotenoids from *Nyctaginalceae*

These compounds are frequently toxic through the inhibition of mitochondrial electron transport chain at the complex I (Gutman et al., 1970). Because of these characteristics, in this thesis additional experiments were
carried out to prove that the methanolic root extract of *B. diffusa* and boeravinone G, at the concentrations used in the study, did not have any toxic effects. The cytotoxicity was assessed quantitatively by both MTT and LDH assays. No decrease was observed in the cell viability and no increase of LDH release when the cell lines were incubated in the presence of either the methanolic root extract of *B. diffusa* or boeravinone G. Moreover, the lack of toxicity of the methanolic root extract of *B. diffusa* was also demonstrated by the Comet assay, since the extract, administered alone (i.e. in absence of damage induced by H₂O₂) did not affect the DNA integrity.

Collectively, these results suggest that the methanolic root extract of *B. diffusa* and boeravinone G are neither cytotoxic nor genotoxic in the Caco-2 cells. Accordingly, in the existing literature, an interesting study is cited aiming at establishing the “toxophore” of rotenoid molecules, discovering that both the prenyl-derived ring attached at the ring D and a dimethoxy substitution on the
ring A are the essential requirements for their toxicity (Crombie et al., 1992) (Figure 29). Fortunately, both these features are missing in B. diffusa rotenoids.
6.0 CONCLUDING REMARKS

In conclusion, the results obtained in this thesis demonstrate that the methanolic root extract of *B. diffusa* possesses spasmolytic activities on intestinal smooth muscles. This effect seems to be due to the presence of boeravinones (which activity depend on their structure and clearly highlight the crucial role of ring B) and the involvement of extracellular calcium and/or L-type calcium channels. Further, the methanolic root extract of *B. diffusa* also exerts antioxidant and genoprotective activity in both chemical and cell (intestinal Caco-2)-based assays. Boeravinone G, H and D appear to be the major responsible of the antioxidant activity, with boeravinone G playing a major role. In the light of the importance of antioxidant activity in the treatment or prevention of gut disorders and since our experiments were performed on isolated intestinal cells, it is suggested that the antioxidant activity here reported could explain, at least in part, the traditional use of this Ayurvedic remedy in treatment of gastrointestinal disorders. These
boeravinones, particularly boeravinone G, might be considered as lead compounds for the development of drugs potentially useful against those pathologies whose aetiology has been related to ROS-mediated injuries. The relatively simple chemical structure of boeravinones and the preliminary structure-antioxidant activity relationships presented here should be helpful in this task.

Moreover, it has been shown that nonprenylated rotenoids isolated from *Boerhaavia diffusa* (boeravinones) could be identify as a new class of promising BCRP (ABCG2) inhibitors. The boeravinones (G and H in particular) as interesting BCPR inhibitors, are able to counter the phenomenon of drug resistance induced by anticancer drug. Hopefully, taking advantage of parallel information obtained for flavone derivatives, chemical modifications on their simple planar structures could pave the way for the development of even more potent MDR modulators.
Finally, the lack of cytotoxicity of these compounds makes them good candidates for *in vivo* tests as adjuvants in antitumor chemotherapy.
7.0 LITERATURE


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Appendix
$^1$H and $^13$C NMR data of 9-O-Methyl-10-hydroxyccocineone B (12). 500 MHz, CD$_3$OD.

<table>
<thead>
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<th>$^1$H mult., $J$ in Hz</th>
<th>$^1^3$C mult.</th>
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</thead>
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<tr>
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</tr>
<tr>
<td>4</td>
<td>7.05, d, 7.8</td>
<td>116.2, d</td>
</tr>
<tr>
<td>4a</td>
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<td>147.7, s</td>
</tr>
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<td>6.06, s</td>
<td>94.2, d</td>
</tr>
<tr>
<td>6a</td>
<td></td>
<td>153.6, s</td>
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<td>9a</td>
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<td>56.0, q</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>132.2, s</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>155.5, s</td>
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$^1$H and $^13$C NMR data of boeravinone G (5). 500 MHz, CD$_3$OD.

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<td>4a</td>
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$^1$H and $^{13}$C NMR data of boeravinone H (4). 500 MHz, CD$_3$OD.

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<td>6.46, s</td>
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$^1$H and $^{13}$C NMR data of boeravinone I (15). 500 MHz, CD$_3$OD.

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$^1\text{H}$ and $^{13}\text{C}$ NMR data of boeravinone J (16). 500 MHz, CD$_3$OD.

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