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Effects of a plant extract from Ruptiliocarpon caracolito on the growth and differentiation of P19 mouse embryonal carcinoma cells

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Effects of a plant extract from *Ruptiliocarpon caracolito* on the growth and differentiation of P19 mouse embryonal carcinoma cells

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ABSTRACT

Present data indicate that the *Ruptiliocarpon caracolito* extract exerted significant anti-proliferative activity against P19 EC cells, in the absence of toxicity and significant alterations in the differentiation status of the cells. Extracts prepared from the bark of the *Ruptiliocarpon caracolito* tree have been reported to possess significant anti-proliferative activities against the European corn borer and some strains of fungi. It has been also reported that the plant extract contains some novel triterpenoids (spirocaracolitones) (MacKinnon, 1995). Plant-derived triterpenoids have been reported to display anti-proliferative activity against numerous cancer cell lines (reviewed by Setzer and Setzer, 2003). Modulating the rate of proliferation in malignant cells may have implications in future cancer therapies. Moreover, *in vitro* systems are thought to be useful for primary stages of screening anti-tumor substances. The focus of this study was to determine whether the proliferation of P19 EC cells would decrease in response to *Ruptiliocarpon caracolito* extract and whether the anti-proliferative activity, if observed, would be accompanied by alterations in the cell differentiation status. According to the data obtained in the present study, the plant extract exerted a significant anti-proliferative activity against the P19 EC cell line. This activity was exhibited in a dose-related fashion. Amongst the doses tested (5-70μg/ ml), concentrations ranging from 30-70 μg/ml showed a significant anti-proliferative activity ($P<0.001$). The highest growth reduction activity of the extract was noticed at a concentration of 50μg/ml of culture medium. Viability studies indicated that the decrease in cell proliferation was not secondary to the toxicity of the extract. Western blot and immunofluorescence experiments demonstrated that *Ruptiliocarpon caracolito* extract let the cells differentiate into neurons with no significant alterations in structural and functional

properties of the neurons. The structural and functional properties were determined in terms of the expression level and distribution of class III β-tubulin and ChAT enzyme, respectively. Moreover, the differentiation status of the P19 EC cells was not significantly altered by the *R. caracolito* extract, as determined by microscopic visualization of three differentiation markers, in the cells exposed to the extract in comparison to the untreated cultures (controls).

ABBREVIATIONS

Ach	Acetylcholine hormone
AchE	Acetylcholineesterase
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CDM	Complex differentiation medium
ChAT	Choline acetyltransferase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
cRABP	Cytoplasmic retinoic acid binding protein
CY3	Indocarbocyanine
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
EC	Embryonal carcinoma
ECB	European corn borer
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol bis 2-aminoethylether N′, N′, N′ tetraacetic acid
ES	Embryonal stem
EtOH	Ethanol
ext	Extract
FCS	Fetal calf serum
GABA	γ-aminobutyric acid
GAD	Glutamic acid decarboxylase
IF	Immunofluorescence
KDa	Kilodaltons
mAChRs	Muscarinic acetylcholine hormone receptors
MAP	Microtubule-associated protein
mRNA	Messenger ribonucleic acid

MTs	Microtubules
MTT	4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
MWs	Molecular weights
NeuN	Neuronal nuclei
NGF	Nerve growth factor
OD	Optical density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer saline
R. caracolito	*Ruptiliocarpon caracolito*
RA	Retinoic acid
RAR	Retinoic acid receptor
RAREs	Retinoic acid response elements
RXR	Retinoid X receptor
SCa	Slow component A
SCb	Slow component B
SD	Standard deviation
SDS	Sodium Dodecyl sulfate
St	Standards
VAChT	Vesicular acetylcholine hormone transporter
VGAT	Vesicular GABA transporter
WCB	Whole cell buffer
βAPP	β-amyloid precursor protein

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CHAPTER ONE

INTRODUCTION

The present study is set out to monitor the biological activities of a plant extract from the bark of *Ruptiliocarpon caracolito* tree. Objectives of this study are to monitor the effects of the extract from the bark of this ethnobotanical tree on proliferation and RA-induced neuronal differentiation of P19 embryonal carcinoma cells.

1.1 P19 Murine Embryonal Carcinoma Cells

Teratocarcinomas are developed in some strains of mice by transplantation of early embryos from uterus to extra-uterine sites (Stevens, 1970). P19 embryonal carcinoma (EC) cells are derived from such a mouse teratocarcinoma. Here, to induce teratocarcinoma, egg cylinders from a 7-day mouse embryo were transplanted into the testis of a 2 to 4 months old male. P19 cells were established directly from tumors developed in this male mouse. Tumors were removed as they reached 1 to 2 cm in diameter, and introduced into culture following collagenase treatment. P19 cells have euploid male normal karyotype 40:XY and they do not contain any gross genetic abnormalities (McBurney and Rogers, 1982). These embryonal cells are able to incorporate in the embryo and contribute to all tissues following injection to an embryo at the blastocyte stage (Rossant and McBurney, 1982).

1.1.1 P19 EC Cells in Culture

Many embryonal carcinoma cells differentiate to a variety of cell types if kept at high density and aggregated *in vitro* (McBurney, 1976; Martin and Evans, 1975) or if exposed to defined serum free medium (Darmon *et al.*, 1981).

Aggregated P19 cells differentiate only into small amounts of extraembryonic endoderm-like cells, although under appropriate conditions these embryonic stem cells can be maintained as exponentially growing cells which consist of undifferentiated cells (Edwards and McBurney, 1983; Jones-Villeneuve *et al.*, 1982). However, P19 cells differentiate to a variety of cell types if exposed to non-toxic concentrations of a number of chemicals. Retinoic acid (RA) and dimethyl sulfoxide (DMSO) are the most effective compounds which can induce differentiation at non-toxic concentrations (reviewed by McBurney, 1993).

RA at concentrations of 10^{-5} to 10^{-7}M induces neurons and astroglia cells while at concentration of ~$5x10^{-8}$M it induces fibroblast-like cells (Jones-Villeneuve *et al.*, 1982). DMSO at concentrations of 0.5%-1% (v/v) promotes the development of epithelial cells containing cytokeratins in small aggregates (each aggregate formed from fewer than 50 cells) and muscle cells containing muscle-specific myosin in large aggregates (each aggregate formed from more than 100 cells) (McBurney *et al.*, 1982; Campione-Picardo *et al.*, 1985_a; Smith *et al.*, 1987). Differentiated cells in DMSO-treated P19 cultures also express high levels of smooth muscle α-actin protein (Rudnicki *et al.*, 1990_a). In later stages of differentiation these cells also differentiate into skeletal and striated frequently beating cardiac muscle cells (Skerjanc, 1999; Rudnicki *et al.*, 1990_b; reviewed by McBurney, 1993). P19-derived cardiomyocytes are able to express a complete set of functional ion channels to generate action potentials (van der Heyden *et al.*, 2003_b).

Obviously, the rhythmic beating of cardiac myocytes demonstrates the existence of a fully functional contraction apparatus in cardiac cells differentiated from P19 EC cells (reviewed by van der Heyden and Defize, 2003_a). Mesoderm-derived cardiac and skeletal muscle cells can also be differentiated from P19 EC cells by co-culturing them with one of several established visceral-endoderm cell lines (END-2) (Mummery *et al.*, 1991). Efficient muscular differentiation of aggregated P19 cells in culture suggests that these cells may be used as a model system to study induction of somite myogenesis (Angello *et al.*, 1997).

Observations show that RA and DMSO have different cellular targets to induce differentiation and DMSO does not inhibit the formation of neurons in cells exposed to both retinoic acid and DMSO (McBurney *et al.*, 1982). Several experimental uses of this cell line in culture show its capability to participate in many normal differentiation pathways.

There are considerable experimental advantages derived from P19 EC cells including the relative ease of their growth and differentiation *in vitro*, which are likely similar to those occurring during normal embryogenesis. These advantages make this cell line a suitable model system to investigate the effect of different factors on growth and/or differentiation of mammalian cells in culture. It is also reported that a particular concentration of chemicals with effects on the growth and differentiation of P19 cells in culture shows almost the same effect on the central nervous system (CNS) *in vivo*. Moreover, this established cell line is evaluated as a good model system to assess the effect of different toxicants on cell viability and differentiation (Seeley *et al.*, 1998_a) and there is evidence showing the use of this system toward this objective (Seeley *et al.*,

1998_b).

1.2 Neuronal Differentiation of P19 EC Cells

1.2.1 Retinoic Acid and Neuronal Differentiation

RA is present in specific areas of the nervous system and it is able to influence neural patterning and stimulate neurite outgrowth in these areas (reviewed by Maden and Holder 1992). *In vitro* experiments show that RA induces neuronal differentiation of embryonal stem (ES) cells (Bain *et al.*, 1995) and EC cells. Moreover, *in vitro* experiments show that RA induces neuronal differentiation even in EC cells that behave as nullipotent cells and do not differentiate under any other conditions tested (Strickland and Mahdavi 1978: Jetten *et al.*, 1979). However, experiments on mouse stem cells show that for efficient induction of neurons as in P19 cells some protocol modifications are needed (Bain and Gottlieb, 1998). There is cumulative evidence for the inductive effect of RA on cholinergic properties of neurons as well as for increased survival of this population of neurons induced by RA. These data reveal that RA is able to increase activity and transcription of choline acetyltransferase (ChAT) and to enhance expression levels of acetylcholine hormone (ACh) and vesicular ACh transporter (VAChT) in established cell lines and primary preparations *in vitro* (Casper and Davies, 1989; Wuarin and Sidell, 1991; Berse and Blusztajn, 1995; Matsuoka *et al.*, 1989; Berrard *et al.*, 1993; Kobayashi *et al.*, 1994; reviewed by Malik *et al.*, 2000). There are different suggestions for the mechanism of RA on cholinergic phenotype expression. These suggestions vary from the direct activation of retinoic acid receptors (RAR) (Pedersen *et al.*, 1995) to activation of some mediators such as growth or neurotrophic factors with ability to

induce cholinergic phenotype (Wang and Halvorsen, 1998; Michikawa *et al.*, 1993).

1.2.2 Postulated Mechanism of Action of RA

There are various receptors, enzymes and proteins involved in RA mechanism of action. Presence of a cytoplasmic binding protein for RA (cRABP) has been reported in some EC cell lines (Jetten and Jetten, 1979). This protein has been proposed to control the concentration of free RA in the cell (reviewed by Leid *et al.*, 1992). cRABP is one of the proteins involved in RA mechanism of action. However, direct effect of RA appears to be mediated by two classes of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Each of these classes of receptors consists of three isotypes of α, β and γ (Zelent *et al.*, 1989; reviewed by Leid *et al.*, 1992). Prior t o neuronal differentiation, all these receptors act as ligand-inducible transcription enhancer factors and modulate gene expression by binding to RA response elements (RAREs) in promoter regions of target genes (reviewed by Glass *et al.*, 1991). To induce neuronal differentiation, there are a few different kinds of naturally occurring retinoids known to activate nuclear retinoic acid receptors. These retinoids include all-trans retinoic acid, and 9-cis retinoic acid (reviewed by Leid *et al.*, 1992). RARs are activated by both all trans-RA and 9-cis-RA, while RXRs respond to 9-cis-RA only (Heyman *et al.*, 1992; Allenby *et al.*, 1993). Experiments show that RAR and RXR bind as dimers to RAREs (reviewed by Malik *et al.*, 2000). The RAR-RXR heterodimer binds much more efficiently and selectively to RAREs than either RAR or RXR homodimers. Moreover, this heterodimer binds to specific response elements that are different from the response elements related to any of the homodimers (Leid *et al.*, 1992; reviewed by Chambon, 1996).

Investigations monitoring the mechanism of RA action on P19 cells show that neuronal differentiation of these cells is induced by cooperation of selective ligands for both RAR and RXR (Horn *et al.*, 1996). P19 EC cells express RAR-α and RAR-β genes (reviewed by McBurney, 1993). Cells with mutation in RAR-α do not differentiate in presence of RA (Pratt *et al.*, 1990) and transfection of these mutants with wild-type human RAR-α restores their RA sensitivity (Kruyt *et al.*, 1992). Moreover, expression of cRABP gene is shown to be reduced following RA treatment in cells with truncated RAR-α (Malý and Dráber 1992). These data suggest the importance of this type of receptor for RA-induced differentiation of P19 EC cells. Additionally, all three forms of RXR-α, –β and -γ mRNAs are detected in undifferentiated P19 cells and they respond differently after exposure to RA (Bain and Gottlieb, 1994). Genomic footprinting analysis of P19 cells exposed to RA show that binding of liganded RAR/RXR to RAREs plays a critical role in recruitment of other factors to their respective elements in the particular promoter region (Dey and Ozato 1997). Experiments also show that, although addition of RAR ligands is able to activate transcription by itself, addition of two ligands related to RAR and RXR both (simultaneous activation of both receptors) leads to synergic activation of transcription (Minucci *et al.*, 1997). In addition to the role of nuclear retinoic acid receptors in RA mechanism of action, there are several enzymatic pathways to synthesize as well as metabolize RA which may play crucial roles in the profound effects of retinoids (Napoli *et al.*, 1995). For example an enzyme induced by RA, CYP26, (Ray *et al.*, 1997) shows an active role in early neuronal development of P19 EC cells probably through generation of specific active RA metabolites (Sonneveld *et al.*, 1999). There is also evidence showing the induction of cRABP gene in P19 cells

following RA treatment (reviewed by Leid *et al.*, 1992).

These data postulate that RA induces neuronal differentiation of P19 cells through cytoplasmic, enzymatic and most importantly, nuclear pathways.

1.2.3 Neurons Differentiated from P19 EC Cells

P19 EC cells differentiate to neurons, astroglia and fibroblast-like cells following exposure to RA and most efficiently at RA concentrations of $5x10^{-7}$M up to 10^{-5}M (Jones-Villeneuve *et al.*, 1982). Axons and dendrites developed from P19 neuronal processes in culture are capable of forming morphologically ((McBurney *et al.*, 1988) and functionally mature synapses (Finley *et al.*, 1996) with electrical properties (Cheun and Yeh, 1991). Moreover, microglia-like phagocytes are observed to be generated in later stages of differentiation (Aizawa *et al.*, 1991) and their presence is dependent on the seeding density of the cells. Astroglia are abundant when P19 cells are induced to neuronal differentiation at a density of $2\text{-}4x10^4$ cells/cm^2 but very few are present at $1x\ 10^4$ cells/cm^2 (Kitani *et al.*, 1997).

Investigations to demonstrate the status of P19 derived neurons *in vivo*, revealed that RA-treated P19 EC cells survive for periods of at least 4 months following transplantation into adult rat striatum. These grafted cells differentiate to fully developed, electrophysiologically functional neurons and glial cells without causing tumor formation in rat brain (Morassutti *et al.*, 1994). Within 2 weeks of transplantation, these neurons are able to respond to glutamate (excitatory neurotransmitter), γ-aminobutyric acid (GABA) and glycine (inhibitory neurotransmitters). These observations indicate that receptors for both excitatory and inhibitory neurotransmitters are expressed in P19 derived neurons differentiated in rat brain (Magnuson *et al.*, 1995). It has also been reported that

oligodendrocytes which are derived from RA-treated P19 cells after transplantation to the brains of neonatal rat pups are capable of developing myelinated axons (Staines *et al.*, 1996). Therefore RA-exposed P19 EC cells may serve as a source of neuronal precursors for transplantation into mammalian CNS. These data also suggest that the mechanisms of P19 cell differentiation are similar to those of CNS and RA-treated P19 EC cells may be used for *in vitro* study of neuronal development and maturation (Morassutti *et al.*, 1994). This suggestion is further supported by the fact that survival of these neurons in culture can be prolonged in serum free growth-conditioned medium (Schubert *et al.*, 1990), which provides enough time for *in vitro* study of development and maturation.

Neurons differentiated from P19 cells in culture contain several different neuronal markers such as neurofilaments, tetanus toxin binding sites (Jones-Villeneuve *et al.*, 1983), NeuN which is a nuclear protein almost exclusively expressed in neurons (Mullen *et al.* 1992), class III β-tubulin as the β-tubulin isotype which is expressed exclusively in the neuronal cytoskeleton (Falconer *et al.*, 1992) and neuron-specific isoform of β-amyloid precursor protein (βAPP) which is a glycosylated membrane-spanning precursor molecule (Hung *et al.*, 1992). It was observed that the latter protein can stimulate expression of acetylcholineesterase (AchE) which is the enzyme responsible for removal of Ach in cholinergic neurons (Sberna *et al.*, 1997).

All these data further support the validity of the suggestions regarding the use of this cell line to study the normal or disturbed events in development and maturation of neurons *in vitro*.

1.2.4 Factors Involved in Neuronal Differentiation

It was reported that RA exerts its differentiation-inducing effect on P19 cells predominantly during the G1 phase of the cell cycle (Mummery *et al.*, 1987_a). Moreover, another study showed that RA induced differentiation of P19 EC cells is accompanied by a large elevation of RAR-α and –β mRNA expression, which is significantly higher in G1 than in S-phase of the cell cycle (Jonk *et al.*, 1992). These observations suppose that RA induces P19 cells to neuronal differentiation in a cell cycle-dependent manner. It has also been observed that only synchronized cells treated with RA for at least 24 hours (h) will express late markers of neuronal differentiation (Mummery *et al.*, 1987_a). As such, this system might provide a good model to study the relationship between growth and differentiation. However, another study assessing the differentiation by a different criterion suggested that RA efficiently induces differentiation of P19 cells regardless of cell cycle status (Berg and McBurney 1990).

Epidermal growth factor (EGF) is a developmental/differential regulatory protein which appears to have a role in differentiation of P19 EC cells. EGF receptor (EGFR) is a cell surface receptor including a cytoplasmic tyrosine kinase whose activity opens a wide range of possible targets and is activated upon binding to EGF. Although a small amount of intracellular EGFRs are present in P19 EC cells, they do not appear to contain any cell surface EGFR. There are observations substantiating the role of EGF in P19 EC cell differentiation. These observations show quite significant increase in the EGFR expression both in mRNA and protein levels starting 2 days after exposing cells to RA (Joh *et al.*, 1992). Furthermore, introduction of human EGFR into P19 cells exposed to EGF stimulates neuronal differentiation of these cells in the absence of RA (den Hertog

et al., 1991). Conversely, blocking the activity of endogenous EGFR curtails the RA-induced differentiation of these cells quite significantly (Wu and Adamson, 1993). All these data suggest the role of tyrosine kinase activation in RA-induced differentiation of P19 cells.

Interactions between individual and groups of cells are shown to be important in regulation of cell proliferation and differentiation in early murine development (Ducibella *et al.*, 1975). Investigations to determine factors with crucial roles in the efficiency of RA-induced neuronal differentiation of P19 EC cells, showed that density of cells in culture is critical in this case. These experiments demonstrated that cultures seeded at very high cell density ($2.5x10^4$ cells/cm^2) are less efficiently differentiated induced by RA (Berg and McBurney 1990). However, increased cell-cell contact may not be responsible for this observation as the number of contacts per cell is not significantly decreased in low density cultures and the neurons readily form contacts with each other even at low culture densities. Moreover, there are studies suggesting the requirement of calcium-dependent cell-cell contact during RA-induced neurogenesis of P19 EC cells (Schmidt *et al.*, 1992). In summary, according to different observations it seems that a combination of factors including seeding density, time of exposure, serum free conditions and concentration of RA determines the efficiency of neuronal differentiation.

1.3 Populations of Neurons Differentiated from P19 EC Cells

P19 neurons are reported to be primarily cholinergic (Kubo, 1989; McBurney *et al.*, 1988) or GABAergic (Staines *et al.*, 1994). The reason for this difference in neuronal populations is still unknown, although observations showed that particular culture conditions and microenvironments are playing crucial roles in this case.

1.3.1 Cholinergic Populations of Neurons

Two crucial enzymes for the function of cholinergic neurons are ChAT and AchE. ChAT catalyzes the formation of Ach in cholinergic neurons (reviewed by Dobransky and Rylett, 2003) and AChE removes Ach in order to allow repolarization to take place (reviewed by Soreq and Seidmans, 2001). P19 neurons show ChAT and AchE activities (Jones-Villeneuve *et al.*, 1983) and they express a high level of ChAT and AchE (McBurney *et al.*, 1988). These data suggest that some neurons differentiated from P19 cells are cholinergic. However, several neuropeptide enzymes of vasoactive agents are detected in endothelial cells (reviewed by Loesch, 2002) and ChAT is one of the enzymes expressed in vascular endothelial cells of small brain vessels (Parnavelas *et al.*, 1985). Since so far there is no report indicating the presence of vascular endothelial cells within the cells differentiated from P19 EC cells, most probably in this case ChAT is expressed within the neuronal cells. P19 neurons express all subtypes of muscarinic acetylcholine hormone receptors (mAChRs) and the expression level of these receptors is increased from days 9-11 of differentiation. These receptors are functional and localized in presynaptic and postsynaptic sites (Parnas *et al.*, 1998). mAChRs are involved in a number of physiological processes such as learning and memory (reviewed by Greenwald and Davis, 1983), neurotransmission and muscle tone (reviewed by Ventura *et al.*, 2002).

Studies also showed that P19 neurons synthesize the excitatory neurotransmitter Ach which increases over the period of incubation for 5-10 days following RA treatment. Presynaptic vesicles observed in P19 neurons look to be round and hollow, which suggests the use of synthesized Ach as the neurotransmitter ((McBurney *et al.*, 1988). Another study showed that synaptogamin, a synaptic vesicle protein essential for

calcium-dependent neurotransmitter release, is expressed both at mRNA and protein levels after day 9 of RA-induced differentiation of P19 cells and Ach release is noted only on or after day 10 (Parnas and Linial 1995).

Parnas and Linial (1995) showed that P19 neurons may display both cholinergic and GABAergic phenotypes which would coexist at days 8-10 of differentiation. Based on their observations, culture density modulates the cholinergic phenotype of neurons. Increasing the culture density from $5x10^4/cm^2$ to $2x10^5 cells/cm^2$ decreases the cholinergic phenotype of neurons from more than 85% to almost 0% (not detectable) in terms of expression of cholinergic markers including ChAT and Ach. In contrast, populations of GABAergic neurons are about 10% at all cell densities which means that this phenotype is not influenced by cell density (Parnas and Linial, 1995). Later on, it was revealed that this modulation is caused by reduction in the expression of ciliary neurotrophic factor (CNTF) receptors in dense cultures (Parnas and Linial, 1997_a). CNTF is known to stimulate cholinergic differentiation of sympathetic neurons in culture (Saadat *et al.*, 1989). It also increases expression of ChAT gene along with a similar increase of ChAT activity in culture (Zinman *et al.*, 1998). P19 neurons possess CNTF receptors located on the cell membrane with a high affinity for their ligand. This neurotrophic factor is shown to enhance the survival and neuronal differentiation of P19 cells (Gupta *et al.*, 1993). Although P19 cells in dense cultures ($6x10^5$ cells/cm^2) do not show a high proportion of cells with a cholinergic phenotype, they are accelerated toward neuronal maturation in several aspects including enhanced expression levels of most synaptic vesicle proteins, increased neurite outgrowth and earlier segregation of axons and dendrites (Parnas and Linial, 1997_b).

There was also another report indicating that more than 85% of P19 derived neurons show a cholinergic phenotype after day 9 of differentiation (Fukuchi *et al.*, 1992). These data suggest that P19 EC cells have a high tendency to differentiate to cholinergic populations of neurons and they acquire the ability to release neurotransmitters after several days of differentiation.

1.3.2 GABAergic Populations of Neurons

Glutamic acid decarboxylase (GAD) is a biosynthetic enzyme of the inhibitory neurotransmitter GABA (reviewed by Wong *et al.*, 2003). There are five monoclonal antibodies that recognize GAD (GAD-1 to-5) (Gottlieb *et al.*1986). It has been observed that GAD transcripts are expressed in undifferentiated embryonal stem (ES) cells and similarly GAD1 and GAD2 transcripts are expressed at very low levels in uninduced P19 EC cells. However, expression levels of these GAD transcripts increase dramatically at 8-10 days following RA treatment (Bain *et al.*, 1993; Bain *et al.*, 1994) with a transcriptional regulation specific for each transcript (Pinal *et al.*, 1997). Interestingly, GAD is co-expressed with μ- and δ-opioid receptors in P19 cells committed to neuronal differentiation. This observation suggests the use of this model system to study relationship between opioid receptors and GABAergic neurons (Chen *et al.*, 1999). Both these opioid receptors are present in mammalian brain cells (Fowler and Fraser, 1994).

Other investigations demonstrated that P19 neurons possess high affinity uptake for the inhibitory neurotransmitter GABA (McBurney *et al.*, 1988). Expression of GABA, GAD, GABA-transaminase (Staines *et al.*, 1994) and GABA receptors (Reynolds *et al.*, 1994) are also observed in neurons differentiated from P19 EC cells.

There is also evidence showing the presence of functional glutamate receptors in

P19 neurons. Glutamate is the major excitatory neurotransmitter in mammalian central nervous system (Ray and Gottlieb, 1993; Turetsky *et al.*, 1993; Morley *et al.*, 1995). Additionally, P19 neurons form both excitatory and inhibitory synapses mediated by receptors for glutamate and GABA or glycine respectively (Finley *et al.*, 1996). In addition to glutamate receptors, P19 neurons release glutamate which, in contrast to Ach, is quite enhanced in dense cultures ($6x10^5$ cells/cm^2) in comparison to sparse cultures ($1x10^5$ cells/cm^2). However, efficiency of GABA release is not affected by culture density (Parnas and Linial, 1997).

Besides GAD and GABA, vesicular GABA transporter (VGAT) is another essential component of GABAergic neurons which is expressed in P19 neurons (Ebihara *et al.*, 2003). All these data suggest that at least a population of neurons differentiated from P19 EC cells is GABAergic.

1.4 Microtubule Organization in Neurons

1.4.1 Structural and Functional Approach

Microtubules (MTs) are one of the main constituents of the cytoskeleton of eukaryotic cells. These constituents determine the shape and structure of the cells and play critical roles in many cell functions such as cell motility, mitosis and intracellular transport (reviewed by Maccioni and Cambiazo, 1995). Tubulin is the main component of microtubules which is composed of two tubulin polypeptides, α and β. α and β subunits associate to form the cylindrical wall of hollow microtubule filaments. Each subunit has different isotypes with most variability in the carboxyl-terminal region which is distinctive for each isotype. These isotypes are encoded by small heterogeneous multi-

gene families. So far, each α- and β- tubulin subunits of mouse microtubules have been identified to belong to six different isotype classes. There is also evidence for selective utilization of specific isotypes in specialized cells (Lopata and Cleveland, 1987; Oakley and Oakley, 1989; reviewed by Joshi and Cleveland, 1990).

Neurons are the data–processing elements of the nervous system. During neuronal differentiation, cells transform their mitotic and cytoplasmic microtubule arrays and undergo extensive rearrangements to generate neuronal processes whose functions include neurite outgrowth and establishment and maintenance of neuronal morphology. Neuronal processes are composed of axons and dendrites. Axons lack ribosomes and tubulin is exclusively synthesized in the cell body. Axons elongate from the pre-existing MTs by transportation of tubulin predominantly in the anterograde direction and at appropriate rate toward the tip of growing ends. The majority of tubulins are transported by slow axonal flow (reviewed by: Baas, 1997; Kobayashi and Mundel, 1998).

The MT system is composed of MTs together with microtubule-associated proteins (MAPs). The carboxy terminus of β-tubulin which is responsible for the differences in β-tubulin isotypes is involved in MAP binding. There is evidence suggesting an important role for MAPs in regulating the differential stability exhibited by neuronal MTs. These data suggest that β-tubulin isotypes are determinant in the recruitment of particular MAPs to specific microtubules and therefore in controlling the diverse functions exhibited by this system (Burgoyne *et al.*, 1988; reviewed by: Laferrière *et al.*, 1997'; Kobayashi and Mundel, 1998).

1.4.2 Class III β-tubulin

Class III β-tubulin, also known as cβ4, is one of the β-tubulin isotypes in the mammalian brain which is exclusively expressed in neurons (Frankfurter *et al.*, 1986; Moody *et al.*, 1989; Lee *et al.*, 1990_a). This isotype differs by approximately 10% in amino acid sequence from other β-tubulin isotypes present in the brain (reviewed by Sullivan, 1988). Experiments showed that class III β-tubulin is selectively phosphorylated on a C-terminal residue during neurite outgrowth of differentiating neuroblastoma cells *in vitro* or during incubation of mouse brain neurons with $^{32}PO_4$ in culture (Gard and Kirschner, 1985; Eddé *et al.*, 1989; Lee *et al.*, 1990_b). These data suggest a selective regulatory role for class III β-tubulin and its post-translational modifications during neuronal differentiation and neurite elongation.

Unphosphorylated class III β-tubulin is reported to be the first neuron-specific lineage marker (Fanarraga *et al.*, 1999). *In vitro* experiments on rat and chick embryo neuroblasts indicated that during the process of differentiation, class III β-tubulin is expressed during or immediately after a final mitotic event (Memberg and Hall, 1995; Moody *et al.*, 1989; Lee *et al.*, 990_a). This observation further suggests that class III β-tubulin represents one of the very first neuronal markers.

Experimental observations showed that regeneration of dorsal root ganglion following crush injury of the distal sciatic nerve coincides with a dramatic increase in β-III mRNA and shift of β-tubulin isotype balance toward class III β-tubulin (Moskowitz and Oblinger, 1995; Moskowitz *et al.*, 1993). Similar changes are also observed in β-tubulin isotypes transported along regenerating sensory axons (Hoffman and Luduena, 1996).

There are investigations revealing the role of β-tubulin isotypes in the dynamic

status of microtubules (Panda *et al.*, 1994). Moreover experiments showed that microtubules made from αβIII tubulins polymerize twice as rapidly as whole brain tubulins in presence of tau or MAP 2. The same experiment in presence of glycerol and $MgCl_2$ instead of tau or MAP2 showed the greater extent of αβIII polymerization in comparison to whole brain tubulins with no significant effect on the speed of polymerization (reviewed by Ludueña, 1993). It was also reported that distribution and expression levels of class III β-tubulin reflect the maturation degree of developing mouse brain neurons (Menezes and Luskin, 1994). These data suggest that detection of class III β-tubulin is a good marker to determine the degree of neuronal differentiation and neurite outgrowth even at initial stages of differentiation.

Although low levels of β-III tubulin are expressed in the Sertoli cells of the testis (Lewis and Cowan, 1988; Frankfurter *et al.*, 1986), it does not appear to undergo phosphorylation in these cells. However, many of class III β-tubulin subunits are phosphorylated at serine ^{444} during neurite outgrowth and elongation. Theses data suggest a regulatory role for this posttranslational modification of β-III tubulin in MTs polymerization of neurons (Ludueña *et al.*, 1988; Lee *et al.*, 1990_b; Alexander *et al.*, 1991).

Investigations detecting the presence of β-tubulin isotypes in colchicine-labile and colchicine–stable microtubules (resistance of microtubules against colchicine as a microtubule depolymerizing agent) revealed the sub-cellular sorting of two β-tubulin isotypes in neurons differentiated from P19 EC cells. According to these investigations on day 2 of P19 EC cell differentiation class III β-tubulin is preferentially sorted into colchicine-labile MTs whereas class II β-tubulin is preferentially sorted into colchicine-

stable MTs. Although by day 3 of differentiation which coincides with evident neurite outgrowth in developing neurons, β-III tubulin is detected in colchicine-resistant microtubules (Laferrière and Brown, 1996; Falconer *et al.*, 1992). Additionally, treatment of differentiating P19 EC cells with alkaloid taxol, which promotes assembly of MTs to stable MT polymers, alters the normal sub-cellular sorting of β-III tubulin. Taxol treatment enhances the polymerization and posttranslational modification of β-III tubulin during neuronal differentiation of P19 EC cells (Laferrière and Brown, 1995). These results suggest that different tubulin isotype compositions may lead to different polymerization characteristics and/or different posttranslational modifications.

1.5 Choline Acetyl Transferase as a Cholinergic Phenotype Marker

ChAT is the enzyme responsible for the synthesis of neurotransmitter Ach (Nachmansohn and Machado, 1943) from choline and acetyl-CoA (reviewed by Tuček, 1993). This enzyme is selectively expressed in cholinergic neurons and serves as a cholinergic marker. Although ChAT is encoded by a single gene, it shows polymorphism with multiple transcripts which are expressed by alternative splicing of noncoding exons and differential utilization of the same gene. As observed in human and primates, 6 transcripts are identified from which 4 are translated to the same 69 kilodalton (kDa) protein and the 2 other ones are translated to 82 and 74 kDa proteins (reviewed by Dobransky and Rylett, 2003). While most of the 69 kDa protein is cytosolic, the 82 kDa enzyme is mostly localized to the nucleus (Resendes *et al.*, 1999). Nuclear localization of the 82 kDa ChAT would argue against its involvement in Ach synthesis that occurs in the

cytoplasm of nerve terminals. Since it is unclear if Ach synthesis would occur in the nucleus, the functional role of the nuclear form of the enzyme remains to be elucidated (reviewed by Dobransky and Rylett, 2003). Nevertheless, experiments indicated the detection of just a very low level of 82 kDa enzyme in human and it appears that the major translation products of ChAT-transcripts possess the molecular weight (MW) of 69 kDa (Misawa *et al.*, 1997). Both forms of human ChAT are posttranslationally modified by phosphorylation which causes enhanced catalytic activity of the enzyme (Dobransky *et al.*, 2000). Phosphorylation of ChAT enzyme was also observed in other species such as rat (Schmidt and Rylett., 1993). Additionally, there is another report indicating the presence of 2500 and 6000-nucleotide (nt) ChAT gene transcripts in human brain and neuroblastoma cells which encode 68 kDa and 27 kDa ChAT enzymes respectively, although the 27 kDa protein does not show enzymatic activity for synthesis of Ach (Lorenzi *et al.*, 1992; Grosman *et al.*, 1995).

ChAT is shown to have cytoplasmic and membrane-bound forms, although the latter form seems to be present at the range of just 10-20% depending on the species. This membrane-bound form is postulated to constitute part of a presynaptic complex (Benishin and Carroll, 1983; reviewed by Rylett and Schmidt, 1993), whereas the cytoplasmic ChAT is detected in the cell bodies of cholinergic neurons (Sang and Young, 1998).

ChAT appears to exist in multiple isoforms with quite different MWs in various species. Investigations demonstrated a wide range of MWs for this enzyme (table 1). Considering the large body of literature investigating ChAT isoforms, this enzyme is represented in different forms with MWs which are mostly in the range of 27-34 kDa, 50-56 kDa, 63-68 kDa or 71-73 kDa depending on the species. However, investigations do

Table 1. ChAT isoforms detected in different species.

Species	Molecular Weights	Refs
Human (brain)	69, 74 and 82 kDa	Reviewed by Dobransky and Rylett, 2003
	27 and 68 kDa	Lorenzi *et al.*, 1992
Rat (brain)	28-29 kDa, 50-51 kDa and 73-75 kDa	Badamchian and Carroll, 1985
Bovine (brain)	63, 68 and 73 kDa*	Hersh *et al.*, 1984
Squid (head ganglia)	125 kDa	Hussain and Mautner, 1973
	27 and 56 kDa	Polsky and Shuter, 1976
Drosophila (head)	13, 53, 67 and 75 kDa	Slemmon, 1989; Slemmon *et al.*, 1982; Slemmon *et al.*, 1991
Catfish (brain)	34 kDa(major) and 68 kDa (minor)	Su *et al.*, 1980
Pig (brain)	68 kDa	Eckenstein Thoenen, 1982
Nematode (*Caenorhabdititis elegans*)	54 and 71 kDa**	Rand and Russel, 1985
Mouse (brain)	28, 50 and 66 kDa	Bulloch *et al.*, 1994

*63 and 68 kDa proteins turned to 73 kDa isform only in presence of proteolytic enzyme inhibitors. This observation suggests that 63 and 68 kDa proteins are proteolyzed forms of the native one (73 kDa) (Hersh *et al.*, 1984).

**71 kDa form of the protein has a tendency to convert to a smaller MW species (54 kDa).

not always report identical MWs for ChAT isoforms even in the same species. These discrepancies might be attributed to the gel electrophoresis inaccuracy, although this would not be expected to be more than ±3 kDa. However, there is evidence showing that ChAT is prone to proteolysis which could be partly avoided by protease inhibitors (Hersh *et al.*, 1984). As such, differences in reported ChAT MWs might be due to proteolysis during extraction process while different extraction methods could affect proteolysis ending up to different size fragments as reported. Moreover, the presence of dimeric form of this enzyme has been also reported. However, observations suggest that the dimeric form of this enzyme is most probably formed during the experimental procedures and do not exist in the native state (Rossier J, 1976).

1.5.1 Axoplasmic Transport

A bulk of proteins in neurons will be transported to axons following synthesis in body. This motion is named axoplasmic transport and occurs in two rates. Slow axoplasmic transport (approximately 0.1-10 mm/day) represents the movement of cytoplasmic proteins and fast axoplasmic transport (approximately 50-400 mm/day) represents the movement of membranous organelles. Slow kind of transport has been shown to have two different velocities designated as slow component A (SCa) and slow component B (SCb). SCa (approximately 0.1-1 mm/day) includes proteins such as tubulins and SCb (approximately 5-10mm/day) consists of some other proteins such as glycolytic and soluble enzymes (Brady and Lasek, 1981; reviewed by: Brown, 2000; Shah and Cleveland, 2002).

Following synthesis in the perikaryon, ChAT is transported along the axon to the nerve ending by slow axoplasmic transport (Fonnum and Malthe-Sørenssen, 1973;

Dziegielewska *et al.*, 1976). As demonstrated in sciatic nerves of the normal mouse of various ages from 2-36 weeks, ChAT moves along the axon with a velocity ranging from 0.2±0.1 mm/day to 3.5±0.2mm/day. This velocity decreases as mouse grows older (Jablecki and Brimijoin, 1975). Although ChAT axoplasmic transport seems to be mostly slow, it shows quite different velocities in various species or nerves. For example ChAT is shown to transport at velocity of 15mm/day in rat vagus and 5mm/day in rat hypoglossal nerve (Frizell *et al.*, 1970), which are different from each other and from ChAT transport velocity in mouse nerves which is in the range of 0.1-3.7mm/day.

All these observations support the fact that following synthesis in cell body, ChAT is transported along the axon to its site of action (nerve terminal) by slow axoplasmic transport and may be localized anywhere from the cell body to the presynaptic nerve terminal.

1.6 *Ruptiliocarpon caracolito*

1.6.1 *Ruptiliocarpon caracolito* Tree and Its Natural History

Ruptiliocarpon caracolito (*R. caracolito*) tree (figure 1-A) belongs to a unique American species of the family Lepidobotryaceae with close similarities to the monotypic African genus *Lepidobotrys*. However, because of its similarities to the wood and floral parts of the *Trichilia* genus it was originally thought to belong to the Meliaceae family. *R. caracolito* was previously known as a tree belonging to a family present only in Africa. However in 1994, Hammel and Zamora discovered it quite unexpectedly near Golfito and in the Osa peninsula, humid lowland tropical rainforests located in Costa Rica, South America. As such, this tree was recently cited as an "unfamiliar tree" in the National

Geographic (Weintraub, 1994).

Hammel and Zammora named this tree as *Ruptiliocarpon caracolito* because of its most characteristic features. "*Ruptilis*" means irregularly splitting. "Carpon" means fruit and "caracolito" means small snail referring to the shape of fallen, horny endocarp. As such, this name is a combination of Latin, Greek and Spanish words respectively with "io" as the combining bridge between *Ruptilis* and carpon (Hammel and Zamora, 1993). Appearance of the leaves and fruit of *R.caracolito* plant is shown in figure 1-B.

1.6.2 Chemical Approach

Bioassay guided fractionation of ethanolic extract from the bark of *R. caracolito* led to the isolation of an unusual triterpene which is named spirocaracolitone A (figure 2-A). The structure of this triterpenoid ($C_{44}H_{54}O_{12}$) was resolved by NMR and single crystal X-rays. These experiments revealed the unusual structure of spirocaracolitone in terms of 12-α-methyl group and the novel spiro junction between rings C and D. None of these two features had been observed in other triterpenoids and their occurrence in this plant is an unprecedented phenomenon. There are also several other structural features in spirocaracolitone which are rarely detected in other pentacyclic triterpenoids (MacKinnon *et al.,* 1994). Further investigations led to the isolation of five more compounds which are closely related to spirocaracolitone A. These compounds are named spirocaracolitones B-F. Two of these compounds, spirocaracolitone D and E, are shown in figure 2-B and C. As suggested, it appears that all these compounds are highly oxidized derivatives of the friedelin family of triterpenes (figure 2-D) (Mackinnnon et al., 1997_a).

1.7 Biological Activities of *Ruptiliocarpon caracolito*

There are investigations demonstrating the inhibitory effect of *R. caracolito* extract on the growth of European corn borer (ECB) as well as on malarial activity and fungi growth. According to these investigations, the majority of spirocaracolitones isolated from the bark of the plant possess growth inhibitory effect, although the magnitude of the effect was greater in some types of spirocaracolitones. The following next three sections present a brief review of these bioassays.

1.7.1 Insect Bioassays

Insect bioassays were elicited by screening the effect of the lyophilized ethanolic extract as well as pure spirocaracolitones isolated from the extract against ECB (*Ostrinia nubilalis*), a robust economic insect. The tested compounds were incorporated into the diet of ECB beginning at neonate stage at concentrations of 5 and 50ppm, followed by recording the activity of some biological parameters in comparison to controls. These parameters were factors such as (1) larval weight and death, (2) percentage and days to pupation, (3) percentage of pupal death, (4) days to and percentage of adult emergence, and (5) adult weight. Spirocaracolitones reduced the larval weight of ECB as one of the parameters tested. The highest reduction effect was elicited by spirocaracolitone D at a concentration of 50ppm (38% reduction in the maximum larval weight). This percentage of growth inhibitory effect was followed by spirocaracolitone C (20%) and A (10.5%). This activity was accompanied by other effects such as prolongation of the development time of ECB to pupation and reduction in pupal and adult weight. However, experiments showed that different spirocaracolitones do not exhibit the same order of activity in all

tested parameters. In other words, a particular kind of spirocaracolitone was not always responsible for the highest activity in different testing parameters. Pure spirocaracolitones showed more significant effect in comparison to the crude extract. Therefore, it seems likely that spirocaracolitones are the principal active ingredients in the extract (MacKinnon, 1995; MacKinnon *et al.*, 1997_a). It is suggested that the ECB growth inhibitory effect of the extract is primarily physiological growth reduction since it does not show antifeedant activity. This suggestion is supported by the fact that ECB chemosensilla is observed to be blocked with soft artificial diets such as diets including plant chemicals. As such, it seems unlikely that feeding behavior of ECB be altered by stimulus of artificial diets (taste or smell). Moreover, it is observed that ECB larvae exhibit little feeding aversion if fed with diets containing plant chemicals (MacKinnon, 1995; Canney and Gardner, 1989). Stages of ECB life cycle is shown in figure 3.

1.7.2 Antifungal Bioassays

Antifungal bioassays have screened the effects of the spirocaracolitones against various strains of *Fusarium*. As observed in these experiments, spirocaracolitones at a concentration of 100ppm caused a significant reduction in the hyphal growth of some *Fusarium* species. This concentration of spirocaracolitones induced 15 to 65% and 49 to 78% hyphal growth reduction, after 24 and 48 hours of exposures respectively. As such, this effect was more pronounced after 48 hours. It was also noted that spirocaracolitone E was mostly responsible for the highest growth reduction effect followed by spirocaracolitone D. These results suggest that natural concentrations of spirocaracolitones in the bark of the plant may provide antifungal protection (MacKinnon, 1995).

1.7.3 Antimalarial Activity

Experiments investigating the antimalarial activity of spirocaracolitones demonstrated a high degree of activity which was mostly exhibited by spirocaracolitone A. However, this antimalarial activity was 20 to 40 times less than gedunin (MacKinnon, 1995). Gedunin is one of the compounds isolated from the bark of neem tree (belonging to the family of *Meliaceae*), which is at least as effective as quinine and chloroquine against malaria (Khalid *et al.*, 1989; MacKinnon *et al.*, 1997_b).

1.8 Traditional Usage of *Ruptiliocarpon caracolito*

The Machiguenga tribes of Manu mix the ash of the burnt bark of *R. caracolito* tree with dried powdered leaf of tobacco that they insufflate. They employ no other bark, and as this tree is quite a rare one they travel an estimated 1000 square kilometers to get the bark (Russo E, MD; personal communication with Dr. Arnason J). The story seems to be an unusual one especially because they are extremely specific about this usage. We are not sure if it is used for a medicinal purpose or it might have a neurobehavioral or any effect like tobacco. As such, the very specific usage of this plant poses an intriguing challenge to explore its possible biological activities.

1.9 Plant-derived Triterpenoids and Anti-neoplastic Potentiality

1.9.1 Triterpenoids

The majority of triterpenes exhibit a polycyclic skeleton which are synthesized from cyclic squalene-2, 3-epoxide. These polycyclic skeletons can be formed of five fused rings which yield to pentacyclic triterpenoids. Some plants contain large quantities of triterpenoids which are believed to function as physiological defendants against herbivores and pathogens (reviewed by: Mahato and Sen, 1997; Connolly and Hill, 2000).

1.9.2 Miscellaneous Activities

There are several investigations demonstrating the biological activities of plant-derived triterpenoids as therapeutics. These experiments revealed several activities such as anti-tumor, anti-inflammatory, antiviral/antibacterial/antifungal/antimalarial and anti-HIV effects. However anticancer and anti-inflammatory activities have been so far the most prominent effects exhibited by triterpenoids (mostly pentacyclic triterpenoids) (Anisimov *et al.*, 1979; reviewed by: Mahato and Sen, 1997; Cichewicz and Kouzi, 2004). Naturally occurring triterpenoids in methanolic extract of the gum resin exudates from *Boswellia serrata* have been reported to have significant anti-inflammatory effects on mice (Huang *et al.*, 2000). Another investigation demonstrated the presence of a positive relationship

Figure 1. Appearance of *Ruptiliocarpon caracolito* plant.

A) Tree (adapted from http://www.stri.org/PMCC/resultados%20de%20flora.htm)

B) Fruit and leaves (adapted from http://www.mobot.org/MBOT /Research/ APweb/ orders/celastralesweb.html)

A)

B)

Figure 2. Structures of some triterpenoids isolated from the bark of *Ruptiliocarpon caracolito* (spirocaracolitones).

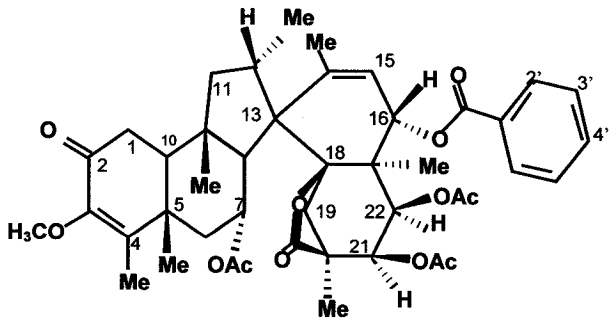
A) Spirocaracolitone A

B) Spirocaracolitone D

C) Spirocaracolitone E (adapted from MacKinon, 1995)

D) Friedelin (adapted from http://www.biosite.dk/staabi/images/friedelin.gif)

A)



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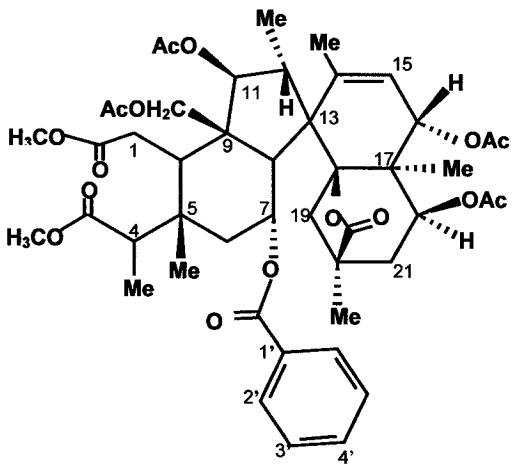

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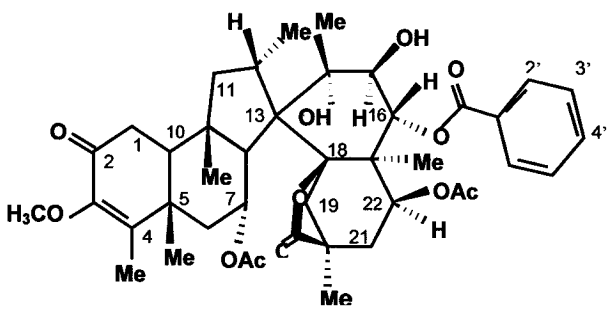

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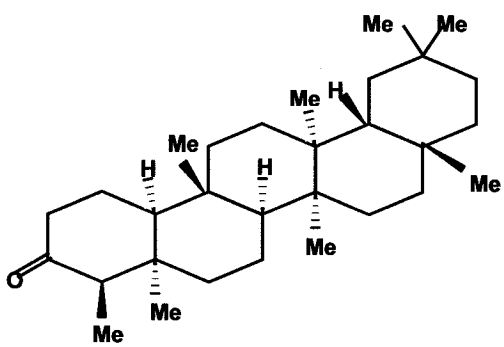

Figure 3. European corn borer (*Ostrinia nubilalis*) at different stages of life cycle.

A) ECB larva (adapted from http://creatures.ifas.ufl.edu/field/ecborer01.htm)

B) Pupa of ECB (adapted from http://ipm.ncsu.edu/photogallery/ECBOR2.JPG)

C) Adult ECB (adapted from http://www.biocont.cz/cornan.html)

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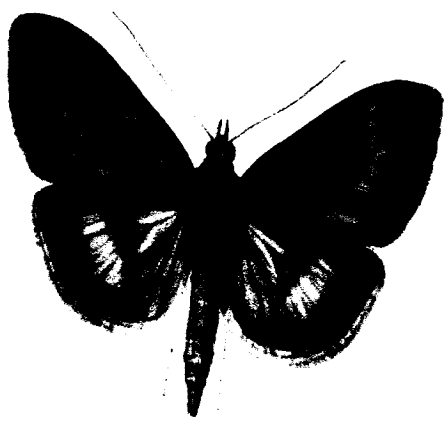

between anti-inflammatory and anti-proliferative effects of two triterpenoids isolated from *Rubus sieboldii*. Anti-proliferative effects in this investigation were caused by the inhibitory action of the testing triterpenoids on desoxyribonucleic acid (DNA) polymerase (Murakami *et al.*, 2002).

1.9.3 Anti-neoplastic Bioactivity

Phytochemicals with anti-tumor activity may be used as primary structures for synthesis of new medications and optimization of their activity. There is mounting evidence for anti-proliferative effects of plant-derived triterpenoids and a large body of literature reflects the potentiality of triterpenoids as anti-neoplastic agents (reviewed by Setzer and Setzer, 2003).

Triterpenoid esters from cranberry fruit, *Vaccinium macrocarpon* (cis- [1] and trans-[2] isoforms of 3-O-p-hydroxycinnamoyl ursolic acid) (figure 4-A) inhibit the growth of tumor cells *in vitro*, with the greatest effectiveness observed on breast tumor cells. Concentrations of less than 20 μM of these triterpenoids (especially cis-isoform) cause 50% growth inhibition (Murphy *et al.*, 2003).

Furthermore, pentacyclic triterpenoids (betulinic, oleanolic and pomolic acids) isolated from the leaves and fruits of *Licania tomentosa* and leaves of *Chrysobalanus jcaco L.* inhibit the growth of human erythroleukemia cell line. More importantly, in addition to inhibiting the growth of sensitive cell lines, these triterpenoids are effective on multi-drug resistant Lucena 1 which is a vincristine resistant human erythroleukemia cell line (Fernandes *et al.*, 2003). Betulinic acid is also known to cause apoptosis in neuroectodermal cells, as well as in cells which are resistant to conventional cytototoxic drugs (Fulda *et al.*, 1997).

Some novel triterpenoids, synthesized as derivatives of oleanolic and ursolic acids, have been shown to induce differentiation in HL-60 leukemia cells. This observation suggests the possible effect of these triterpenoids as cancer preventive agents as they induce neoplasmic cells to differentiate into normal cells (Suh *et al.*, 1997).

Investigations also showed that, from the triterpenoids isolated from the *Sandoricum koetjape* bark, koetjapic acid (figure 4-B) significantly delays tumor promotion in skin-carcinogenesis induced in mouse. This triterpenoid appears to be a promising chemopreventive agent for skin-carcinogenesis (Ismail *et al.*, 2003).

1.10 Hypothesis

Based on the knowledge provided so far I hypothesized that *R. caracolito* extract will reduce proliferation of mammalian carcinoma cells. A corollary to the central hypothesis may suggest an increase in the tendency of cells to go toward the process of differentiation.

1.11 Objectives

To address this hypothesis, I have focused on the following objectives:

1. Monitoring the proliferation and viability of P19 embryonal carcinoma cells in presence and absence of the extract from the bark of *R. caracolito.*
2. Morphological and quantitative comparison of the rate of RA-induced neuronal differentiation in P19 EC cells whether or not exposed to *R. caracolito* extract. This comparison has been performed using both structural and functional criteria.

Figure 4. Plant-derived triterpenoids with anti-neoplastic activity.

A) Cis (1) and Trans (2) 3-O-p-hydroxycinnamoyl ursolic acid a triterpenoid ester isolated from cranberry fruit, (adapted from Murphy *et al.*, 2003).

B) Koetjapic acid, a triterpenoid isolated from the *Sandoricum koetjape* bark, (adapted from, Ismail *et al.*, 2003).

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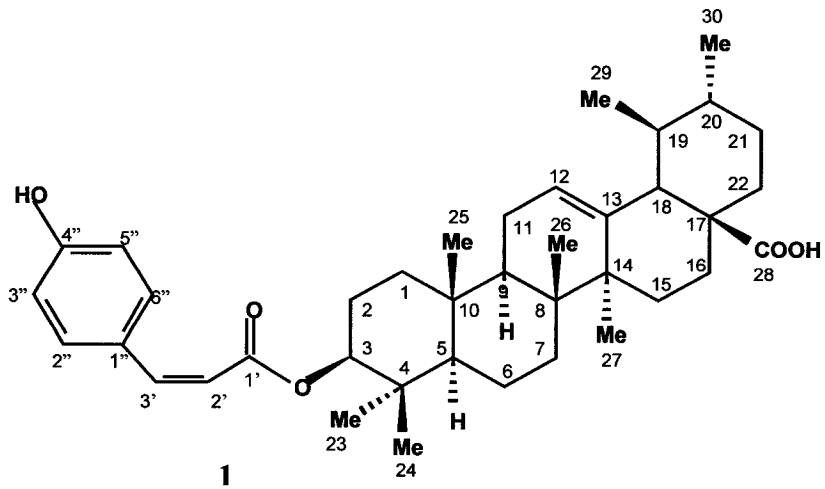
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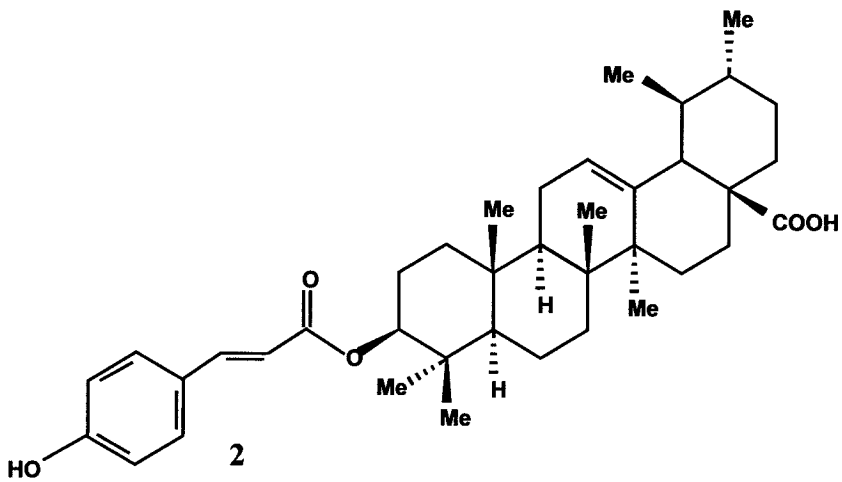
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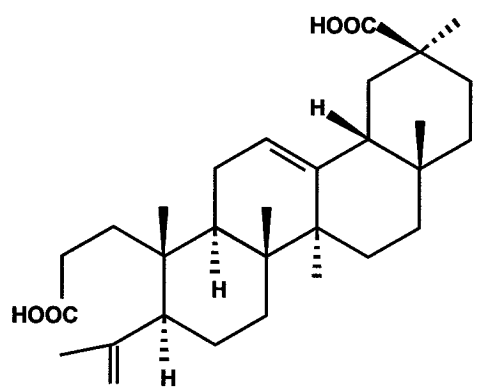
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1.12 Rationale for Experiments

Natural products have long been a fertile source of cure for different kinds of diseases. However, there is a continuing need for development of new drugs by medical and scientific exploration of enormous pools of synthetic and natural products. Moreover, bioactive natural products led to identification of some chemicals as the source of novel agents with pharmaceutical promise. In terms of cancer treatment, there is an urgent need for new chemotherapeutic agents based on the limiting factors such as resistance of cancer cells.

R. caracolito is a plant from Lepidobotryaceae family, which is found in South America (Osa peninsula, located in Costa Rica) and Africa. Insect bioassays showed that extracts from the bark of this plant cause quite a significant reduction in the relative growth of ECB larvae. Bioassays also showed that this extract has significant antifungal and antimalarial activities. The chemicals isolated from the bark of *R. caracolito* and characterized so far are unique triterpenoids, called spirocaracolitones (MacKinnon, 1995; MacKinnon *et al.*, 1994; MacKinnon *et al.*, 1997_a).

A number of triterpenoids isolated from other plants have shown promise as anti-neoplastic agents. This promise is emphasized by the fact that the number of manuscripts abstracted in PubMed about the antineoplastic effects of triterpenoids has increased by more than 24 times from 1987-2001 (reviewed by Setzer and Setzer, 2003). There is also evidence for antifungal, antimalarial and differentiation inducing activities of plant-derived triterpenoids (Anisimov *et al.*, 1979; Umehara *et al.*, 1992; reviewed by: Mahato and Sen, 1997; Cichewicz and Kouzi, 2004).

Differentiation and development of multi cellular organisms require the strict

regulation of cell proliferation (Ducibella et al., 1975). As such it seems likely that any change in the normal pathway of cell proliferation may reflect on cellular differentiation and anti-proliferative agents may be an attractive candidate for modulating cell differentiation. There is evidence for the presence of a dynamic equilibrium between processes of differentiation and proliferation. Some factors are shown to trigger neuronal differentiation by blocking cell proliferation (Campione-Piccardo *et al.*, 1985_b; LoPresti *et al.*, 1992). However, another report provided evidence of the simultaneous inhibition of differentiation and proliferation in embryonal carcinoma cells (F9 EC cell line) exposed to cadmium chloride (Piersma *et al.*, 1993).

Considering all these data, I set out an experimental approach to determine if the extract from the bark of *R. caracolito* has anti-proliferative effects against mammalian carcinoma cells. As the next step, I perused to investigate if there is any relationship between changes in the rate of cell proliferation and differentiation process in my particular biological system. To conduct the later objective, I evaluated the neuronal differentiation in regard to both structural and functional criteria.

Cell lines offer several advantages for biological research due to their relative simplicity and culture reproducibility. As manifested by several criteria P19 EC cell line is a good model system to study cellular proliferation, development and differentiation. As such, I chose this cell line to conduct my experiments.

To assess the cell proliferation as my first objective, I used two standard and extensively used bioassays to monitor cell proliferation and viability. For the second objective, I tried to scrutinize the pattern, localization and expression levels of two different neuronal markers. One of these markers is known to have a crucial role in

neuronal morphology while the other one is determinant of the functional properties of the neurons differentiated from P19 EC cells. As these cells are able to differentiate to multiple cell types, I also conducted experiments to investigate if treating P19 EC cells with *R. caracolito* extract may cause any change in the speed or rate of these embryonal cells to differentiate or go toward the process of differentiation in absence of any differentiation inducing factor.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture

P19 embryonal carcinoma (EC) cell line (McBurney and Rogers, 1982) was the kind gift of Dr. M. McBurney (Cancer Research Center; University of Ottawa). P19 cells were maintained at 37°C in a humid atmosphere with 5% CO2 in Dulbeco's modified essential medium (DMEM; Gibco BRL, Burlington, Ontario, Canada) supplemented with 15% fetal calf serum (FCS; Gibco BRL) and antibiotics (50 U/ml penicillin and 50μg/ml streptomycin; Gibco BRL) (complete DMEM). Cells were passed at sub-confluence every two days using 0.25% trypsin (Sigma Chemical Co., St. Louis, Mo. USA) and 1mM ethylene diamine tetra acetic acid (EDTA; Sigma Chemical Co) in calcium-free and magnesium-free phosphate buffer saline (PBS) (0.08% [w/v] NaCl, 0.002% [w/v] KCl, 0.002% [w/v] KH_2PO_4, and 0.01146% [w/v] Na_2HPO_4). Cells were seeded at a density of $0.5x10^5$/ml of medium and grown as monolayers in 100mm tissue culture dishes.

2.2 Cell Proliferation/Viability Assays

Experiments for the determination of cell proliferation/viability were undertaken using trypan blue dye exclusion and MTT assays as two independent methods. These experiments were conducted at the selected times and data were expressed as the percentage of viable and dead cells in relation to controls.

2.2.1 Trypan Blue Dye Exclusion Assay

Cell proliferation/viability was assayed using the trypan blue dye exclusion assay (Celis, 1994). Trypan blue dye is excluded from viable cells and absorbed by dead cells. In each set of experiments, cells were seeded at the density of $1.8x10^5$ in 60mm tissue culture dishes (density that allowed the untreated controls to grow exponentially for 5 days) in triplicates/day for each set of controls (negative and carrier solvent controls) and testing cultures. At 24 h after plating, the medium was changed with fresh medium containing the extract at concentrations of 5-50μg/ml or its carrier solvent with the same percentage as in the extract. At daily intervals up to day 4 after exposing cells to the extracts, the medium was removed and transferred to a falcon tube. Attached cells were trypsinized and pooled with cells in the tube (removed medium) so that all cells would be included .This mixture was then briefly centrifuged and resuspended. Trypan blue (Sigma Chemical Co) in saline solution was added to an equal volume of resuspended cells (final concentrations, 0.08% trypan blue and 0.45% NaCl). The mixture was then incubated for about 2 minutes at room temperature and a drop of trypan blue-cell mixture was applied to a hemocytometer. Numbers of viable (trypan blue negative) and dead cells (trypan blue positive) were counted separately under the microscope in two different samples for each plate. Counting was duplicated for each sample using the two chambers of hemocytometer.

2.2.2 MTT Cell Proliferation Assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay is an index of cell growth and viability which is simple, rapid and offers a high degree of

precision. This assay is based on the ability of viable cells to reduce MTT (in part by the mitochondrial enzyme succinate-dehydrogenase) from a yellow water soluble dye to a dark blue insoluble formazan product. The latter dye which is produced by MTT conversion is colorimetrically detectable and therefore can be spectrophotometrically quantified with an absorbance which has a highly significant linear relationship with the number of viable cells (Denizot and Lang, 1986; Carmichael *et al.*, 1987). MTT method was used as originally described by Mosmann (1983), in order to measure changes in P19 EC cell proliferation.

To conduct MTT assay, the number of cultured cells were adjusted to a density that allowed the untreated controls to grow exponentially for 5 days. As such $0.4x10^4$ cells were seeded in triplicates for each set of controls (negative control and carrier solvent control) and experimental cultures. In each set of experiments, cells were seeded in 4 parallel 96-well flat bottom plates (Corning Incorporated 3595, Corning, NY, USA) in presence of 100 µl complete DMEM per well. Plates were incubated for 24 h at 37°C in a humid atmosphere with 5% CO2 prior to treating cells with individual concentrations of extracts (30-70 µg/ml) or their carrier solvent (0.05% DMSO).

MTT assay was performed using MTT cell proliferation assay kit (ATCC, Manassas, VA, USA). Standard procedures were followed with some modifications to optimize the precision of the assay for our particular cell line. At daily intervals after exposure of the cells to the testing extract (for 4 days), 10 µl of MTT reagent was added per well and allowed to incubate with cells for 4 h. Incubation was followed by addition of 100 µl of detergent reagent to each and all wells. Plates were swirled gently, sealed with parafilm and left overnight at room temperature in the dark to allow solubilization of

the formazan crystal products. Absorbance of samples was determined at a wavelength of 570 nm and a reference wavelength of 660 nm using microplate reader (Molecular Devices, SpectraMax Plus 384, Sunnyvale CA, USA). The background absorbance of medium in the absence of the cells was subtracted by providing blanks (wells with the same volume of the medium and treated as other wells in absence of cells). Data were presented after determination of the average values from triplicate readings. The cell proliferation indices were calculated by the optical density (OD) value in treated cells divided by the OD value in cell control wells (untreated cells). This assay was conducted in 3 independent experiments and for each set of experiments cell proliferation was monitored at daily intervals up to day 4 after treating cells with appropriate concentrations of extract.

2.3 Induction of Neuronal Differentiation

Neuronal differentiation of P19 EC cells was induced using RA (Jones-Villeneuve *et al.*, 1982) and either N1 medium-supplement (Bottenstein *et al.*, 1980) or complex differentiation medium (CDM) (Macpherson and McBurney, 1995).

RA was obtained from Sigma Chemical Co.; R-2625). N1 medium-supplement was obtained from Sigma Chemical Co. (N-6530). This medium- supplement is composed of: (1) 0.5 mg/ml insulin from bovine pancreas, (2) 0.5 mg/ml human transferrin (partially iron saturated), (3) 0.5 µg/ml sodium selenite, (4) 1.6 mg/ml putrescine, and (5) 0.73 µg/ml progesterone. CDM was prepared as defined by Macpherson and McBurney (1995). Ingredients were as follows: D-MEM [Gibco BRL], 2mM L-glutamine[Sigma Chemical Co.], 1% 100X trace elements B [Mediatch Inc., Cellgro, VA, USA], 1% 100X basal medium Eagle's vitamin solution [Gibco BRL], 1

μg/ml human transferrin [Sigma Chemical Co.], 20 nM hydrocortisone-21-phosphate-glucocorticoid [Sigma Chemical Co.], 10 nM L-carnitine [Sigma Chemical Co.], 100 μM puterscine [Sigma Chemical Co.], 15 nM selenious acid [Sigma-Aldrich Canada Ltd., Oakville, ON], 50 nM cadmium sulfate [Sigma Chemical Co.], 10 μg/ml DL-α-tocopherol [Gibco BRL], 0.2 μg/ml DL-6, 8-thioctic acid [Sigma Chemical Co.], 0.1 μg/ml linoleic acid [Sigma Chemical Co.], 5 μg/ml bovine insulin [Sigma Chemical Co.] and 5 mg/ml bovine serum albumin [Sigma Chemical Co.]).

2.3.1 Differentiation of P19 EC Cells Using N1 medium-supplement

To induce neuronal differentiation in P19 EC cells, cells were plated at a density of $1.66x10^4$/ml of medium (day -1) in triplicates for each set of controls (negative and carrier solvent controls) and testing cultures. Plated cells were maintained as mentioned under section 2.1. Medium was removed on day 0 and replaced with an equal volume of complete DMEM containing 10^{-6} M RA in presence or absence of 50 μg/ml of extract or its carrier solvent (0.05% DMSO). RA was dissolved in absolute ethanol (EtOH) and stored as 10^{-3}M stock at -20°C. RA is light sensitive and, therefore, the stock was kept in foil wrapped tubes. Twenty-four hours later, medium was replaced with the same volume of DMEM containing 1% N1 medium-supplement (day 1) and every 24 hours of culture, half of the medium was refreshed. Cells were kept in culture dishes up to an appropriate time for the experiment and presence of well differentiated, healthy neurons in the culture dishes.

For immunoblot experiments, cells were differentiated in 60mm culture dishes containing 5 ml of medium. For immunofluorescence experiments, cells were differentiated on sterile coverslips (CS) in a 6 wells tissue culture plate (Corning

Incorporated 3516., Corning, NY, USA) containing 2 ml of medium. Neuronal differentiation assay was conducted in three independent experiments.

2.3.2 Differentiation of P19 EC Cells Using Complex Differentiation Medium

Neuronal differentiation experiments were conducted as described under section 2.3.1. However, in these experiments DMEM containing 1% N1 medium-supplement was replaced with CDM.

2.4 Preparation of Cell Extracts

In all cases, whole cell extracts were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein extracts from tissue culture cells were prepared by lysing cells in whole cell buffer (WCB) (Drubin et al., 1985) composed of (25 mM Na_2HPO_4, 400 mM NaCl, 0.5% SDS, 4 mM p-aminoethyl benzene sulfonyl fluoride (pefabloc), 40 μg/ml aprotinin, 40 μg/ml leupeptin, 40 μg/ml pepstatin and ddH_2O). Following a brief rinse in PBS, 200-500 μl of WCB was added to each of the plates and cells were removed by scraping using a rubber policeman and transferred to a microcemtrifuge eppendorf tube. Lysed cells were boiled for 10 min followed by centrifugation at 4°C and 10000 RPM for another 10 min. Supernatants were collected as protein fractions and stored at -80°C when necessary.

2.5 Protein Biochemistry

2.5.1 Protein Concentration Assay

Protein contents of cell lysates were determined using bicinchoninic acid (BCA) protein assay as described by Smith *et al.* (1985). This assay uses the reaction of copper (II) with proteins (reduction of Cu^{2+} to Cu^{1+}) and subsequent reaction of BCA with copper (I) which form a colorimetric compound. Protein content has a highly linear relationship with the color absorbance. As such spectrograph can be used to determine protein concentrations.

BCA assay was conducted as described in the patent of Pierce (Pierce Biotechnology Inc. Rockford, IL, USA; 23227). Briefly, samples and stock solution of bovine serum albumin (BSA) (Omnipur, EM Science, Darmstadt, Germany) standards (2 mg/ml) were diluted in WCB (the same matrix as in the cell lysates). Range of protein standards prepared from the stock solution of BSA was between 0.00-2.00 mg/ml. Aliquots (20 µl) of the standards and diluted unknown protein solutions (samples) were placed in triplicates into wells of a 96-well plate (Corning Incorporated 3595, Corning, NY, USA). The assay was initiated with the addition of 200 µl working reagent to each well. Working reagent was prepared by mixing 50 parts of BCA reagent (Sigma Chemical Co.) with 1 part of copper reagent, 4%$CuSO_4.5H_2O$ (Sigma Chemical Co.) prior to addition to protein samples in each well. The solutions were then incubated for 2 h at room temperature (RT) in order to develop a stable color. Absorbance was measured at 562 nm in a plate reader (SpectraMax Plus 384, Sunnyvale CA, USA)). A standard curve was constructed from absorbance against known BSA concentrations and protein

concentration of each cell homogenate was calculated based on the linear curve of standards.

2.5.2 Polyacrylamide Gel Electrophoresis

To monitor the differentiation quantitatively, each sample of whole cell protein extracts (as described under section 2.4) was solubilized in an equal volume of sample buffer (SB) 2X consisting of 4% SDS, 20% glycerol, 20 mM Tris-Base, 50 mM pure mercaptoethanol and 0.01% bromophenol blue. Solubilized proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), in 5% stacking gel and 12% resolving gel according to the method of Laemmli (1970). Aliquots loaded on the gels were standardized according to their total protein concentrations and equal concentrations of proteins were loaded per well. Proteins in all samples were briefly sonicated and denatured for 5 min at 95°C. Electrophoresis was performed at 100-150V using the minigel apparatus (BIO-RAD, Hercules, California, USA) in electrophoresis running buffer (25 mM Tris Base, 112 mM glycine and 0.1% [w/v] SDS). The gel was run until the bromophenol blue marker reached the bottom of the gel. Molecular weights were estimated from the standards (BIO-RAD, Ontario, Canada) run simultaneously with the samples. To visualize protein bands, gels were either transferred to nitrocellulose membranes or soaked in Coomassie blue (0.1% Coomassie blue in 10% acetic acid and 25% methanol) overnight followed by destaining in 10% acetic acid and 25% methanol until the background was cleared. Gels were then washed with double distilled water and dried overnight between two cellophane sheets (Biodesign Inc. New York, USA).

2.5.3 Western Immunoblotting

To visualize and compare the relative density of the specific protein bands, loaded proteins were separated by SDS-PAGE as described under 2.5.2 (the same amount of total proteins were loaded per lane of SDS-PAGE) and electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell, Keene N.H, USA) according to the method described by Towbin *et al.*(1979). Prior to transfer, gels and membranes were equilibrated in transfer buffer (191.5 mM glycine [BDH, Toronto, ON, Canada], 25 mM Tris-Base (Gibco BRL), 20% [v/v] methanol [Omnisolv EMT^{TM}]). The transblotting cassette was assembled as instructed by BIO-RAD. Transfer was performed at 40V overnight at 4°C. Immunoblotting was performed according to the instructions recommended in the Western Blotting Kit (Amersham Canada, Oakville, Ontario, Canada). To visualize sample lanes, the nitrocellulose membranes containing the transferred samples were stained for 5 minutes with Ponceau Red (0.2% [w/v] Ponceau S dye [Sigma Chemical Co.] in 3% trichloroacetic acid [Sigma Chemical Co.]). Membranes were then destained in PBS (1X) (0.765% [w/v] NaCl, 0.0725% [w/v] Na_2HPO_4 [anhydrous], 0.0212% [w/v] KH_2PO_4 [anhydrous]) and blocked for 1 h in 5% (w/v) Carnation skim milk powder dissolved in PBS-Tween (1:200) (0.05% Tween in PBS [1x]) at room temperature followed by 3x5 minutes wash in PBS-Tween (1:200). Membranes were then incubated for 1 h in primary antibodies and then for another 1 h in the appropriate biotynylated secondary antibodies, each set of antibodies diluted in PBS – Tween (1:200). Rabbit secondary antibodies were diluted in PBS Tween (1:200) containing 5 % (w/v) Carnation skim milk powder. Membranes were rinsed in PBS-Tween (1:200) 3x10 minutes in between and following the two latter incubations. Appropriate controls were performed using the secondary antibodies alone. To permit the

visualization of antigen-antibody complexes, membranes were then incubated in streptavidin-conjugated with horseradish peroxidase (1:5000; Amersham Canada) for 1 h followed by washes 2x10 minutes in PBS-Tween (1:200) and 2x10 minutes in PBS (1X). Detected bands were developed using the chemiluminescence reagents (Amersham, Canada) according to the manufacturer's instruction. Developed bands were either visualized by scanning laser densitometry (BIO-RAD Laboratories, Milan, Italy) or on hyperfilm ECL (Amersham Canada).

2.5.4 Densitometric Analysis

Protein bands were visualized by scanning laser densitometry (BIO-RAD Laboratories, Milan, Italy). Protein levels in each detected band were analyzed quantitatively using Quantity one program (Bio-Rad, Version 4.1.1).

2.6 Cell Fixation

Two fixation methods were employed to optimize the preservation of antigens under study. Simultaneous fixation/extraction and picrate-formaldehyde fixation protocols were applied as follows.

2.6.1 Simultaneous Fixation/Extraction

Simultaneous fixation/extraction of cells was conducted as previously described by Falconer *et al.* (1992). Briefly coverslips displaying a layer of cells were washed 2x30 seconds in PBS (1X) prior to 10 minutes simultaneous fixation/extraction in 2 ml fix/ex solution (4% paraformaldehyde [EM Science], 0.2% Triton X-100 [BDH] in PEM buffer [40 mM PIPES, 2.5 mM ethylene glycol bis 2-aminoethylether N′, N′, N′ tetraacetic acid

(EGTA), 0.5 mM $MgCl_2$], all from Sigma Chemical Co.). This fixative preserves microtubule polymers while permeabilizing the cell membrane. Following fixation, coverslips were rinsed 3x5 minutes in PBS (1X) and incubated 3x4 minutes in 1 mg/ml of sodium borohydride (BDH) solution in PBS (1X). Coverslips were finally washed 3x5 minutes in PBS (1X) and promptly used for immunofluorescence labeling.

2.6.2 Picrate-Formaldehyde Fixation

Paraformaldehyde-picric acid fixation (Stefanini *et al.*, 1967) was used in order to improve cell structure and preserve the maximum cellular antigenicity. According to this protocol, coverslips were briefly rinsed twice in PBS (1X). Coverslips were then fixed for 45 minutes in paraformaldehyde-picric acid fixative. This fixative consists of 100 mM sodium phosphate buffer (pH 6.9) containing 4% paraformaldehyde (w/v) and 14% picric acid (v/v). Following fixation, coverslips were briefly rinsed twice in PBS (1X) and free aldehyde groups were reduced with sodium borohydride (BDH) (1 mg/ml in PBS [1X]) for 3x4 minutes. Cells were then permeabilized for 10 minutes in 0.5% Triton X-100 (BDH) in PBS (1X) and finally rinsed 2x4 minutes in PBS (1X) prior to incubation with antibodies.

2.7 Immunofluorescence Experiments

2.7.1 Indirect Immunofluorescence Staining

To permit the visualization of specific cellular constituents, cells were stained as previously described (Chaly *et al.*, 1984) following fixation. Briefly, cells attached to coverslips were incubated in primary antibody for 45 minutes at room temperature.

Coverslips were then rinsed in PBS (1X) for 15 minutes with three changes of PBS prior to incubation with the appropriate secondary antibody for 45 minutes at room temperature. To visualize DNA, stained cells were immersed for 3 minutes in 1 μg/ml Hoechst 33258 (Polysciences Inc., Warrington, Pennsylvania, USA) diluted 1:3000 in PBS (1X). Secondary antibody and Hoechst incubation were followed by 3x5 minutes washes in PBS (1X). Appropriate controls were performed using the secondary antibodies alone. Finally to retard fluorescence bleaching, coverslips were mounted in a medium consisting of 0.1% p-phenylene diamine (Sigma-Aldrich Canada Ltd.) in 50% glycerol/PBS (v/v), pH 7.0.

2.7.2 Immunofluorescence Microscopy

Fluorescence images were collected with a Zeiss Axiophot microscope equipped with a 50 W Hg burner. Digital images were taken using the Hamamatsu charge-coupled device (CCD) camera (Hamamatsu Photonics K.K. Japan) settled on the microscope. Images were captured with the Metamorph image analysis software system (Version 4.01, 1999, Universal Imaging Corp., USA). Micrographs were edited using PowerPoint (Microsoft).

2.8 Antibodies

2.8.1 Primary Antibodies

The following primary antibodies were used for all immunoblotting and immunofluorescence experiments:

1. **TUJ1**, a mouse monoclonal (IgG) specific for class III β-tubulin (gift from Dr A. Frankfurter) which is the isotype of β-tubulin expressed exclusively in neurons (Frankfurter *et al.* 1986). This antibody was diluted in PBS (1X) 1:5 for immunofluorescence (IF) staining and 1:10 in PBS-Tween (1:200) for immunoblotting experiments.
2. **ChAT**, a rabbit polyclonal IgG (Chemicon, CA, USA), against ChAT which is the catalyzing enzyme for synthesis of Ach neurotransmitter in cholinergic neurons. This antibody was used at 1:500 in PBS (1X) for IF staining and at 1:1000 in PBS-Tween (1:200) for immunoblotting experiments.
3. **DM1A**, a mouse monoclonal IgG (Sigma Chemical Co.) specific for α-tubulin and used at 1:500 dilution in PBS (1X) for IF staining.
4. **A60 NeuN**, Neuronal Nuclei (NeuN), a mouse monoclonal IgG (gift from Dr. M.W. McBurney) specific for a nuclear protein which is exclusively expressed in neurons (Mullen *et al.*, 1992). This antibody was diluted 1:5 in PBS (1X) prior to be used for IF staining.
5. **Troma-1**, a rat monoclonal IgG (gift from Dr. MW. McBurney) specific for EndoA (Brulet, *et al.*, 1980). Endo-A is a cytokeratin (component of intermediate filaments), expressed only in cells undergoing differentiation (Takihara *et al.*, 2000). This antibody was diluted 1:5 in PBS (1X) prior to be used for IF staining.
6. **Lamin A/C** antibody, a mouse monoclonal IgG against lamins A and C (provided by Dr. G. Krohne, Biocenter of the University of Wurzburg, Wurzburg, Germany). Lamins are proteins of nuclear-specific intermediate filaments (reviewed by Holaska, *et al.*, 2002). Lamins A and C are expressed in P19 EC

cells only if they are fully differentiated (Collard and Raymond 1990). This antibody was diluted 1:100 in PBS (1X) prior to be used for IF staining.

7. **SSEA1** antibody, a mouse monoclonal IgM (gift from Dr. MW McBurney) specific for stage-specific embryonic antigen1 (SSEA1) (Solter and Knowles, 1978). SSEA1 is a cell surface antigen and marker for undifferentiated murine embryonal carcinoma cells (Knowles *et al.*, 1978; Fenderson *et al.*, 1993; Andrews *et al.*, 1994). This antibody was diluted 1:10 in PBS (1X) prior to be used for IF staining.
8. **MAN**, a human antiserum to label MAN antigens which are non-lamin constituents of the nuclear envelope in vertebrate cells (Paulin-Levasseur *et al.*, 1996). This antibody was diluted 1:7000 in PBS (1X) prior to be used for IF staining.

2.8.2 Secondary Antibodies

The following biotinylated antibodies were used as secondary antibodies for immunoblotting experiments at the dilutions indicated:

1. Biotinylated-donkey **anti-mouse** (Amersham Canada Ltd.) diluted 1:4000 in PBS-Tween (1:200).
2. Biotinylated-donkey **anti-rabbit** (Amersham Canada Ltd.) diluted 1:4000 in PBS-Tween (1:200) /milk (5%).

The following indocarbocyanine (CY3) conjugated secondary antibodies were used for immunofluorescence staining experiments at the dilutions indicated:

1. CY3-conjugated donkey **anti-mouse IgG** (Jackson Immuno Research Laboratories Inc., Grove, PA, USA), diluted 1:400 in PBS (1X) prior to use.

2. CY3-conjugated donkey **anti-rabbit IgG** (Jackson Immuno Research Laboratories Inc.), diluted 1:400 in PBS (1X) prior to use.
3. CY3-conjugated donkey **anti-rat IgG** (Jackson Immuno Research Laboratories Inc.), diluted 1:400 in PBS (1X) prior to use.
4. CY3-conjugated donkey **anti-mouse IgM** (Jackson Immuno Research Laboratories Inc.), diluted 1:400 in PBS (1X) prior to use.
5. CY3-conjugated donkey **anti-human IgG** (Jackson Immuno Research Laboratories Inc.), diluted 1:400 in PBS (1X) prior to use.

2.9 Preparation of Plant Extracts

Ruptiliocarpon caracolito extract was the kind gift of Dr. John T. Arnason (Department of Biology, University of Ottawa). It was lyophilized and kept in the fridge at 4°C prior to be used in our research experiments.

Ruptiliocarpon caracolito plant materials were collected from a variety of locations in Costa Rica in June 1992, ground up with a Wiley mill and transported to Ottawa University in 95% ethanol. Extract was produced from the bark of the plant by an appropriate extraction process (MacKinnon, 1995).

I chose dimethyl sulfoxide (DMSO) as the carrier solvent because the lyophilized plant extract was found to be more soluble in DMSO. As such by using DMSO as the carrier solvent, we were able to keep the DMSO percentage as low as 0.05% (with no effect on P19 EC cells proliferation), while increasing the concentration of extract (added to cell cultures) up to 70 μg/ml of medium. To apply the plant extract in our experiments the lyophilized extract was resuspended in the appropriate volume of DMSO. To achieve solubilization, the mixture was placed in an eppendorf thermomixer for 4 h at 25°C and

1200 RPM. The mixture was then centrifuged at 3500g for 4 min. The supernatant obtained after centrifugation was used for the experiments following dilution in an appropriate volume of complete DMEM.

2.10 Statistical Analysis

Statistical analysis was performed on SigmaStat software (SigmaStat statistical software, version 3.0, 2003, SPSS Inc, San Rafael, CA, USA). Analysis of variance was carried out using the one-way analyses of variance (ANOVA) in conjunction with Holm-Sidak test. P values less than 0.05 was considered statistically significant.

CHAPTER 3

RESULTS

3.1 Proliferation of P19 EC Cells under Standard Conditions

To monitor the proliferation of P19 EC cells under standard conditions, cells were seeded at a density that allowed an exponential growth for 5 days in a 60mm tissue culture plate. To seed the cells as evenly as possible, $1.8x10^5$ cells were suspended in 5 ml of complete D-MEM prior to plating cells on culture dishes on day 0. Figure 5 depicts the proliferation and viability of P19 EC cells at daily intervals up to day 5 after plating cells in three independent experiments. Each set of experiments was performed in triplicates and the mean and standard deviation between triplicates in each set of data are presented in table 2. As shown in figure 5, P19 cells do not show proliferation on the first plating day. However, it seems that the number of cells almost triples per day between days 1-3. Proliferation slows down after day 3 as cells approach confluency on day 5. Moreover, as shown in figure 5, the proportion of dead cells increases over time. This increase is more pronounced on culture days 3 to 5.

3.2 Effects of the Extract from *Ruptiliocarpon caracolito* on P19 EC Cell Proliferation

3.2.1 Trypan Blue Dye Exclusion Assay

Cell proliferation assays were initiated by dissolving the freeze-dried extract in absolute ethanol (EtOH) as the carrier solvent. In these preliminary experiments effects of the extract dissolved in EtOH were studied at concentrations ranging from 1-10μg/ml

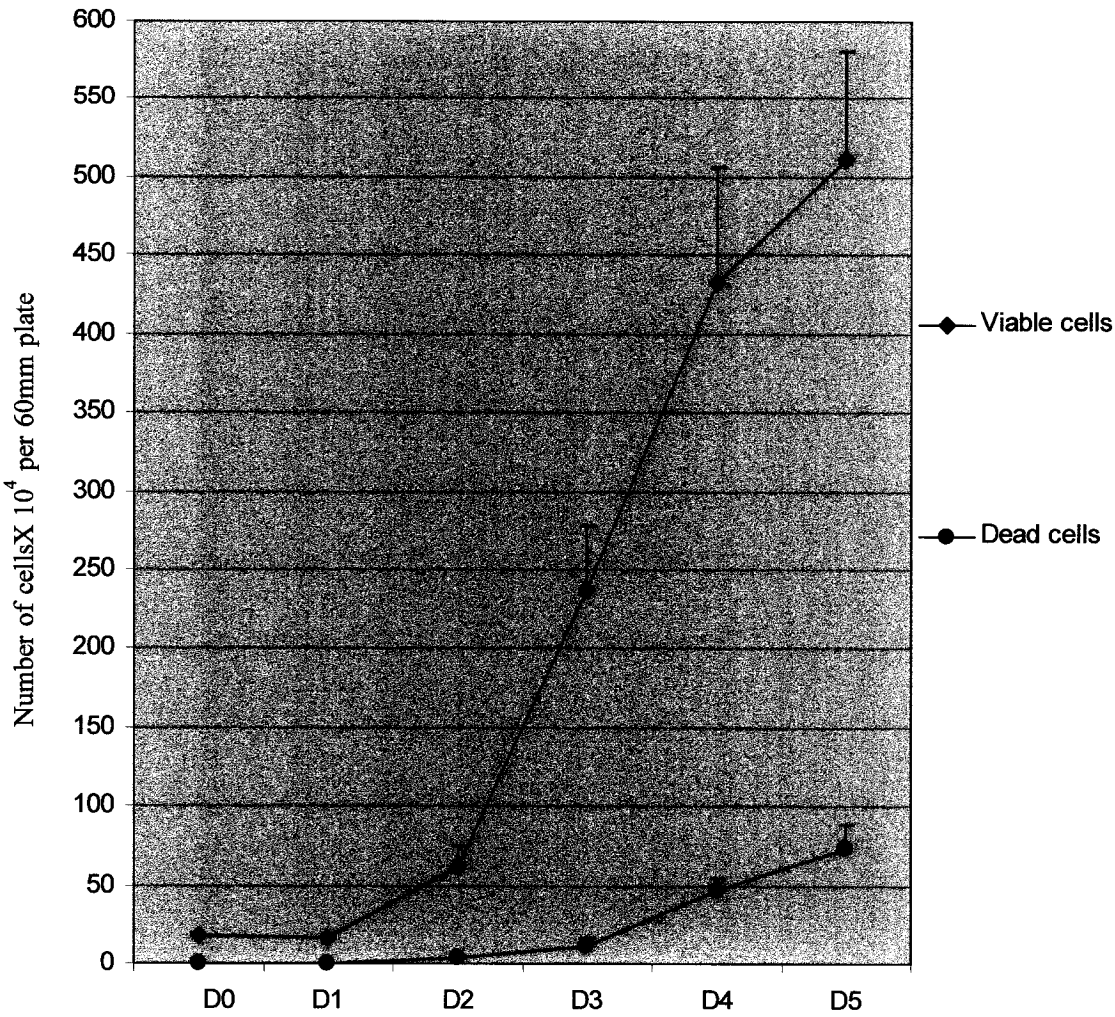
Figure 5. Normal proliferation and viability of P19 EC cells over 5 days in culture.

Cells were plated at a density of $1.8x10^5$ in 60mm culture dishes on day 0. Each data point represents the mean of three independently performed experiments (each in triplicates). Error bars represent the standard deviations among all sets of data (n=9). Abbreviation: D, day.

—◆— Represents viable cells

—●— Represents dead cells

Normal proliferation and viability of P19 EC cells over 5 days in culture



of culture medium. As determined by carrier solvent controls, EtOH even at a percentage as low as 0.025% decreased P19 EC cell proliferation. However, because of the low solubility of the lyophilized extract in EtOH, the percentage of this carrier solvent could not be lowered more than 0.025% (in culture medium). The lyophilized extract exhibited a good solubility in DMSO (as the carrier solvent), allowing to keep the percentage of the carrier solvent in the culture medium as low as 0.05%. This percentage of DMSO did not exhibit any anti-proliferative activity on P19 EC cells as determined by carrier solvent controls. However, low concentrations of extract (5-10 μg/ml of culture medium) did not show any effect on P19 EC cell proliferation/viability (data not shown). As shown in figure 6, *R. caracolito* extract at a concentration of 16 μg/ml of culture medium caused a slight (by the average of 21.67%) decrease in P19 EC cell proliferation. This effect was determined by the average number of viable cells on day 2 after treating cells with the extract in comparison to the untreated cultures (negative controls). However, statistical analysis did not show any significant decrease in P19 EC cell proliferation induced by this concentration (16 μg/ml of culture medium) of the extract ($p>0.05$). Moreover, carrier solvent controls did not exhibit any effect on P19 EC cell proliferation induced by DMSO (0.05%).

Insect and antifungal bioassays on spirocaracolitones isolated from *R. caracolito* extract showed that spirocaracolitones at concentrations of 50 μg/ml and 100 μg/ml exhibit the highest growth reduction effect on insect (ECB) and fungi (*Fusarium* species), respectively (MacKinnon, 1995). Considering these data and the slight effect of *R. caracolito* extract (16 μg/ml of culture medium) on P19 EC cell proliferation, experiments were continued by monitoring the effects of the extract at a concentration of

50 µg/ml of culture medium. These experiments were conducted at daily intervals up to day 4 after exposing cells to the extract. Treatment with *R. caracolito* extract at the concentration of 50 µg/ml of culture medium caused a significant ($P<0.05$) decrease in proliferation of P19 EC cells on days 1-3 after exposure (culture days 2-4) (table 3). In fact, compared to untreated controls *R. caracolito* extract treatment (50 µg/ml of culture medium) resulted in 13.04%-39.91% growth inhibition in P19 EC cells from day 1 to day 4 following treatment (in terms of the average numbers of viable cells in three sets of data). This growth inhibitory effect developed over time from 24 h (30.7%) to 48 h (39.91%) after exposure, whereas on day 3 and day 4 this effect decreased to 27% (significant) and 13.04% (not significant), respectively. The proportion of dead cells was not significantly changed by *R. caracolito* extract in comparison to solvent and negative controls. Moreover, P19 EC cell proliferation and viability were not significantly changed by the carrier solvent of the extract (0.05% DMSO). The effects of *R. caracolito* extract (50 µg/ml of culture medium) on proliferation and viability of P19 EC cells are illustrated in figure 7. This curve shows the effects *of R. caracolito* extract (50 µg/ml of culture medium) over the period of 4 culture days after treatment.

3.2.2 MTT assay

In order to confirm the results obtained from the trypan blue dye exclusion assay, cells treated with *R. caracolito* extract were submitted to MTT assay as described in Materials and Methods. In these experiments, effects of the extract on P19 EC cell proliferation were examined at concentrations ranging from 30 to 70 µg/ml of culture medium and at daily intervals from day 1 to day 4 after exposure. Figure 8 illustrates the proliferation of P19 EC cells in the presence or absence of the extract (30-70 µg/ml

Figure 6. Effects of *R. caracolito* extract on proliferation and viability of P19 EC cells (trypan blue dye exclusion assay).

Cells were plated at a density of $1.8x10^5$ on 60mm plates. *R. caracolito* extract was added to the culture dishes at a concentration of **16 µg/ml** of culture medium 24 hours after plating cells. This extract was dissolved in DMSO as the carrier solvent. Effects of the extract on cell proliferation and viability were examined on day 2 after treatment using trypan blue dye exclusion assay. Each data point represents the average of 2 independently performed experiments (each in triplicates). Error bars on the graph show the SD among all sets of data (n=6). **Ext** (extract) represents the data related to the cells treated with *R. caracolito* extract. **Control** represents controls or untreated cultures, and **DMSO** represents cultures treated with the same percentage (0.05%) of carrier solvent (DMSO) as in the extract (carrier solvent control) 24 hours after plating cells. Abbreviations: V, viable; D, dead; T, total which represents total number of cells (viable cells + dead cells).

Effects of *R.caracolito* extract on proliferation and viability of P19EC cells

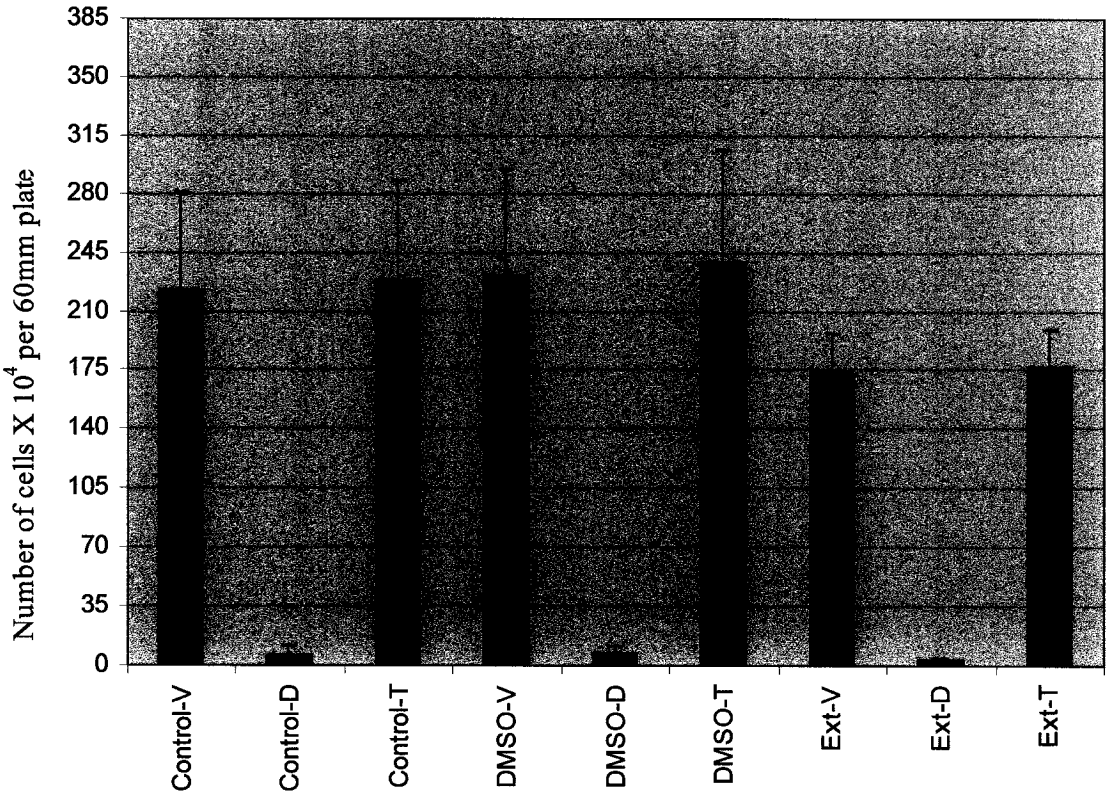


Figure 7. Treatment of P19 EC cells with *R. caracolito* extract results in cell growth inhibition (trypan blue dye exclusion assay).

Cells were treated with *R. caracolito* extract (50 µg/ml of culture medium) or its carrier solvent (0.05% DMSO) 24 hours after plating $1.8x10^5$ cells in 60mm plates. Number of viable and dead cells in each testing and control cultures (each in triplicates) were monitored separately at daily intervals during four days of incubation in presence of the extract or DMSO as well as in untreated cultures. Experiments were performed in triplicates and each data point represents the average of the three sets of data. Error bars on the figure represent the SD between triplicates. **Control** represents untreated cultures, **Extract** represents cultures exposed to the extract and **DMSO** represents cultures treated with the same percentage (0.05%) of carrier solvent (DMSO) as in the extract (carrier solvent controls).

—◆— Represents viable cells in control cultures

—■— Represents viable cells in cultures exposed to DMSO (0.05%)

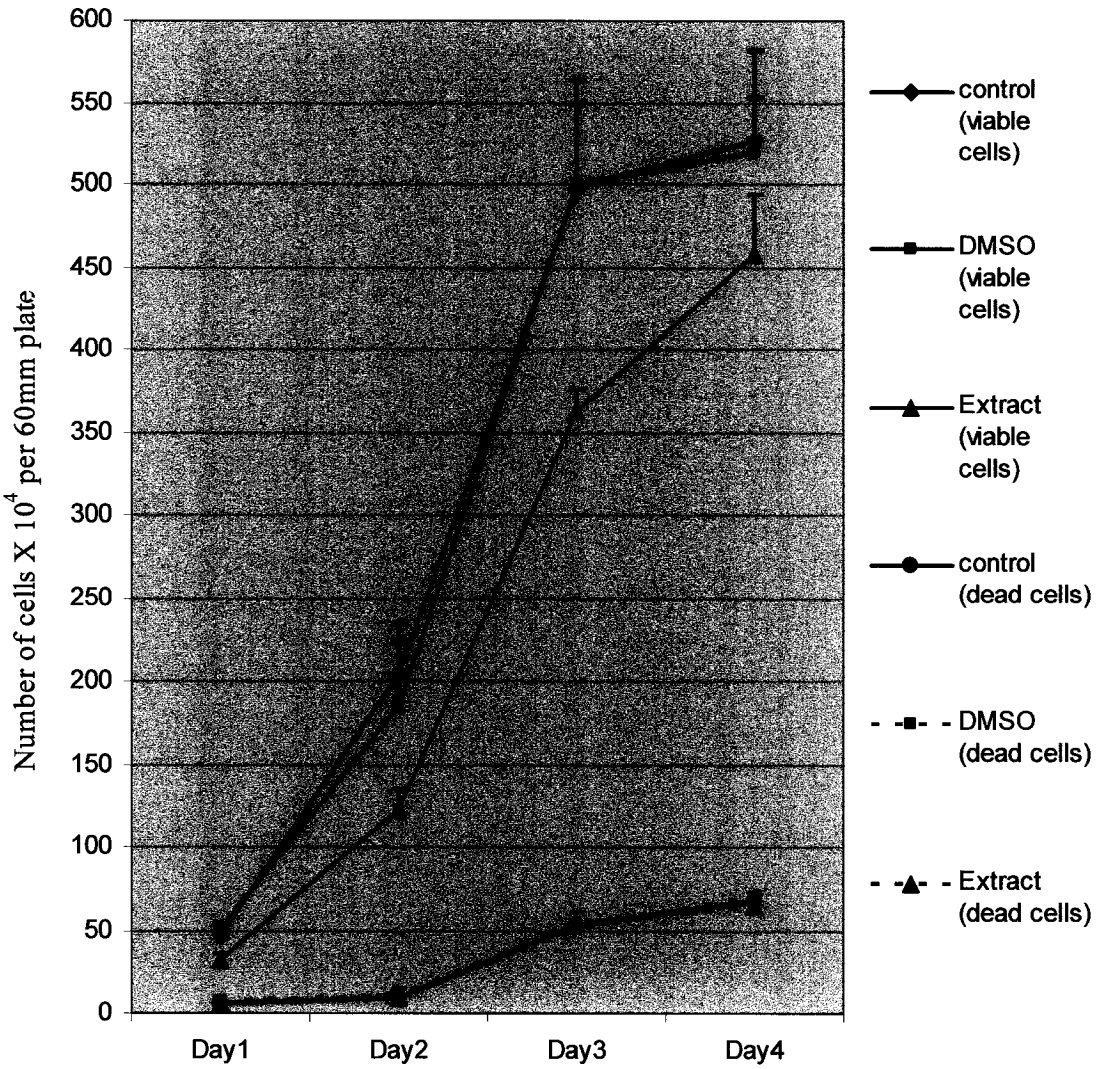
—▲— Represents viable cells in cultures exposed to the extract (50 µg/ml of culture medium)

—●— Represents dead cells in control cultures

–■– – Represents dead cells in cultures exposed to DMSO (0.05%)

–▲– – Represents dead cells in cultures exposed to the extract (50 µg/ml of culture medium)

Inhibitory effects of *R. caracolito* extract on the growth of P19 EC cells



Days after exposing cells to the exract

of culture medium) or its carrier solvent in three independently performed experiments. Treatment of P19 EC cells with *R. caracolito* extract caused a statistically significant reduction in cell proliferation on days 2 to 4 after exposing cells to the tested concentrations (table 4). As shown in figure 8, the anti-proliferative effects of the extracts develop over time from day 1 to day 3 and decrease after day 3 of exposure. Table 5 summarizes the dose dependent effects of the extract at daily intervals, up to day 4 after exposure, and figure 9 compares the anti-proliferative activity of different concentrations of the extract on day 3 after exposure. As depicted in this figure, among the concentrations tested, the highest anti-proliferative activity was observed at a concentration of 50 μg/ml of culture medium. As such, *R. caracolito* extract used in all further experiments was employed at a concentration of 50 μg/ml of culture medium. As shown in figure 8, 0.05% DMSO did not induce any significant change in cell proliferation or viability as determined by carrier solvent controls. The data obtained from the MTT assay support the findings from the trypan blue dye exclusion assay, indicating the inhibition of growth by *R. caracolito* extract. The present findings demonstrate for the first time a significant anti-proliferative effect of *R. caracolito* extract on P19 EC cells.

3.3 Effects of *Ruptiliocarpon caracolito* Extract on the Structure of Neurons Differentiated from P19 EC Cells

3.3.1 β-III Tubulin Expression Level

Class III β-tubulin is the β-tubulin isotype which is exclusively expressed in neurons (Frankfurter *et al.*, 1986). As such, in present experiments, the expression level

Figure 8. *R. caracolito* extract decreases the proliferation of P19 EC cells (MTT assay).

Results of the MTT assay at daily intervals from day 1 to day 4 following treatment of P19 EC cells with *R. caracolito* extracts are shown. Cells were treated with *R. caracolito* extract (70, 50, 40 and 30 µg/ml of culture medium; abbreviated as Ext 70, Ext 50, Ext 40, Ext 30 in the figure) and 0.05% DMSO (carrier solvent control). In each set of experiments untreated cultures were assayed as negative controls. The cell proliferation indices were calculated as the optical density (OD) values in treated cells divided by the OD values in negative controls (untreated cells). Therefore, cell proliferation indices in negative controls are considered as 1. Each data point represents the average of three independently performed experiments. Error bars on the graph show the SD among all sets of data (n=3).

– ■ – · Represents the ratio of cell proliferation in cultures exposed to DMSO (0.05%)

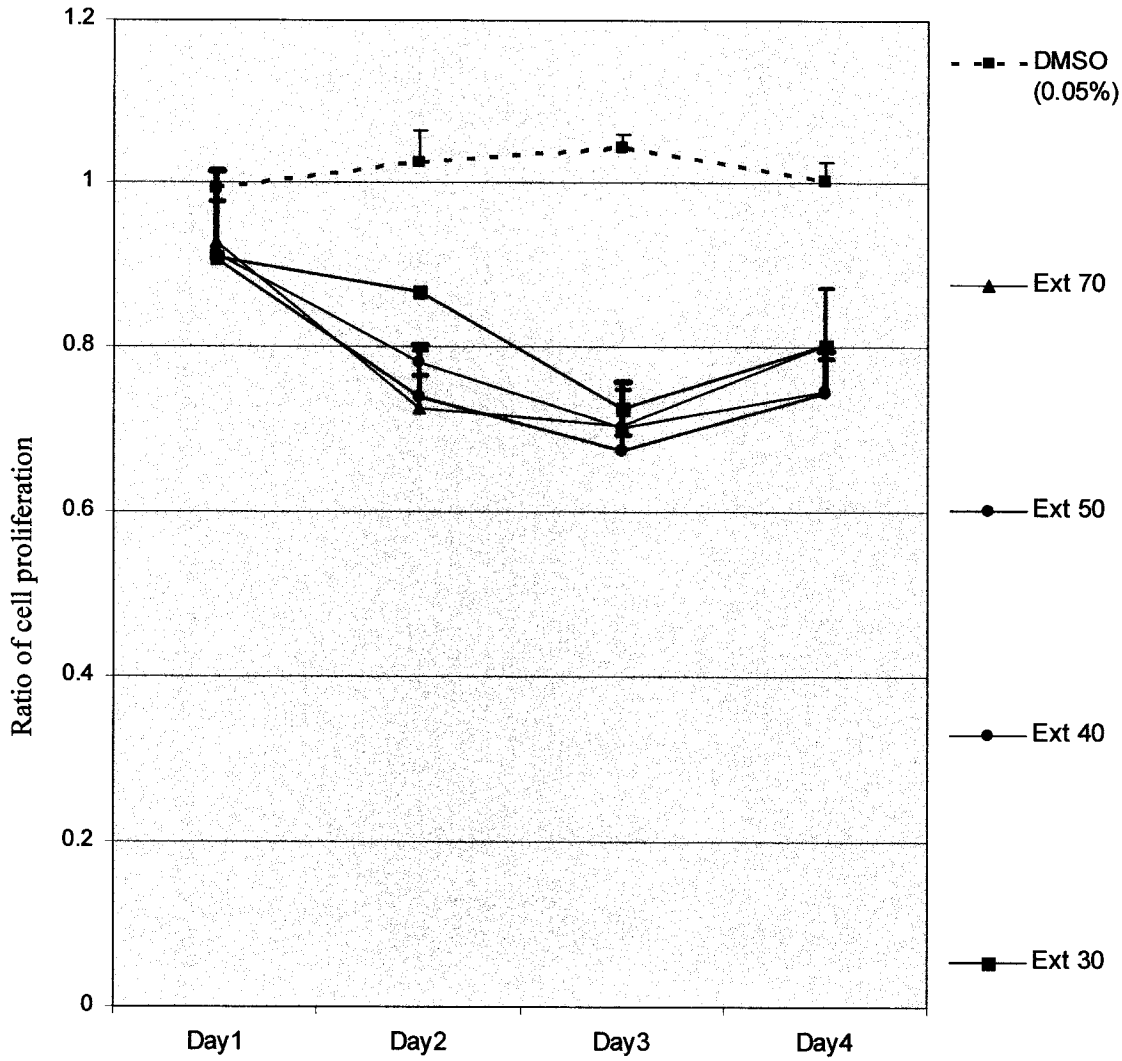
—▲— Represents the ratio of cell proliferation in cultures exposed to the extract (70 µg/ml)

—●— Represents the ratio of cell proliferation in cultures exposed to the extract (50 µg/ml)

—●— Represents the ratio of ell proliferation in cultures exposed to the extract (40 µg/ml)

—■— Represents the ratio of cell proliferation in cultures exposed to the extract (30 µg/ml)

Anti-proliferative effects of *R. caracolito* extract on P19 EC cells

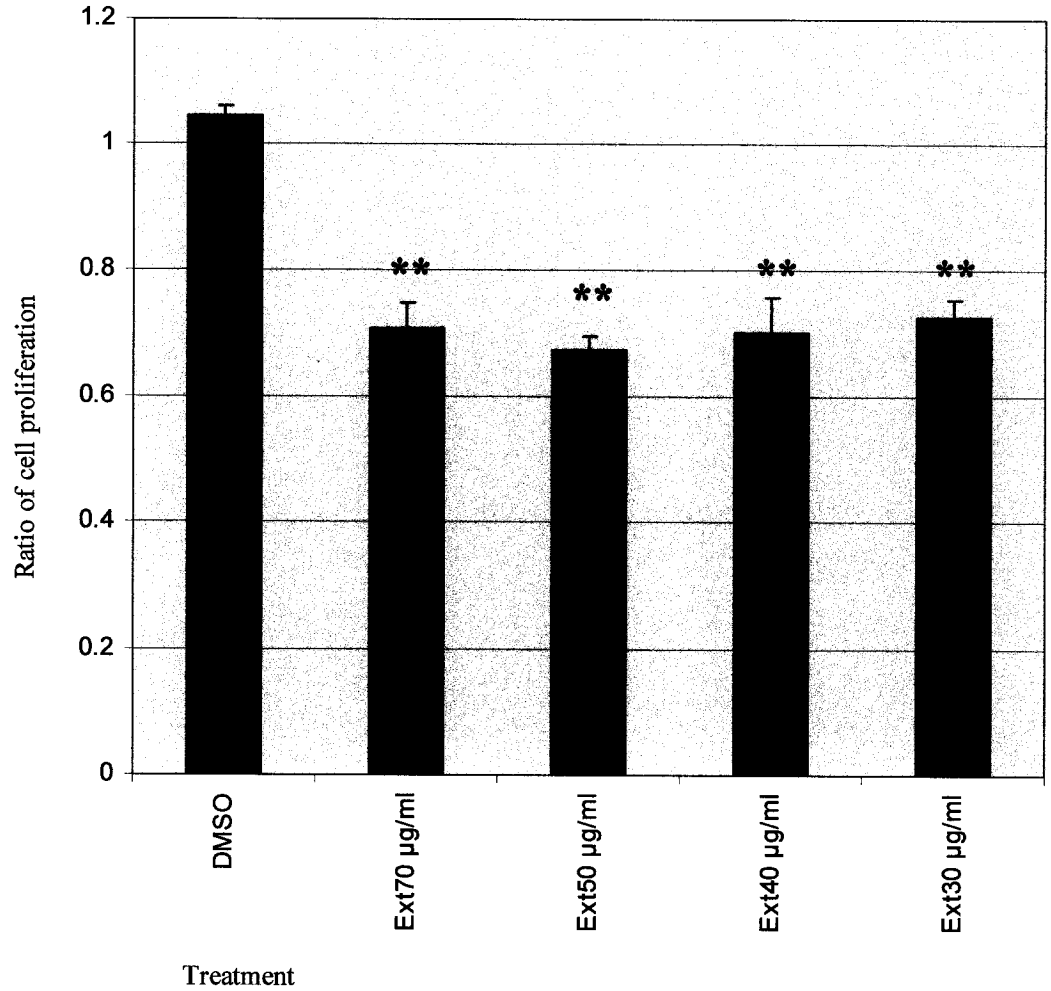


Days after exposing cells to the extract

Figure 9. Treatment of P19 EC cells with *R. caracolito* extract at concentrations ranging from 30 to 70 µg/ml results in significant reduction in cell proliferation (MTT assay).

Results of the MTT assay are shown on day 3 following treatment of P19 EC cells with *R. caracolito* extract (at concentrations of 70, 50, 40 and 30 µg/ml of culture medium) and its carrier solvent, DMSO (0.05%). Each data point represents the average of three independently performed experiments. Ratio of cell proliferation is relative to negative controls (untreated cells), which is considered as 1. **Ext** (extract) represents the cultures exposed to the extract. **Control** represents untreated cultures and **DMSO** represents cultures exposed to DMSO (0.05%). ** Represents the significant growth reduction of the cells ($P<0.001$). Error bars on the graph show the SD among all sets of data (n=3).

Reduction in P19 EC cell proliferation induced by different concentrations of *R. caracolito* extract on day 3 following treatment



of class III β-tubulin was monitored in order to investigate the possible effects of *R. caracolito* extract on neuronal differentiation of P19 EC cells and/or the ability of differentiating cells to generate neuronal processes. To accomplish this, expression levels of β-III tubulin were quantitatively compared in testing and control cultures. To conduct these experiments, P19 EC cells were exposed to different culture conditions prior to be submitted to SDS-PAGE and western blot experiments (in three independent experiments).

Two different sets of standards were loaded on gels for SDS-PAGE. Standards used for gels stained in Coomassie Blue were **prestained broad range** standards **(St)** consisting of myosin, **198** kDa; β-galactosidase, **115** kDa; bovine serum albumin, **93** kDa; ovalbumin, **49.8** kDa; carbonic anhydrase, **35.8** kDa; soybean trypsin inhibitor, **29.2** kDa; lysozyme, **21.3** kDa; aprotinin, **6.4** kDa. Standards used in the gels to be transferred to nitrocellulose membrane were **low range biotynylated** standards **(St)** consisting of phosphorylase B, **97.4** kDa; bovine serum albumin, **66.2** kDa; ovalbumin, **45** kDa; carbonic anhyrase, **31** kDa; soybean trypsin inhibitor, **21.5** kDa; lysozyme, **14.4** kDa.

The set of samples to be tested were prepared by differentiating P19 EC cells to neurons in presence or absence of the extract or its carrier solvent. Differentiation was induced by exposing the cells to RA (10^{-6} M) on day 0 (24 h after seeding cells) followed by incubation in N1 medium-supplement from day 1 to day 4 prior to harvesting on day 4 of differentiation. To monitor the effects of the extract on the β-III tubulin expression level, P19 cells were differentiated, as mentioned above, in 3 separate culture dishes. On day 0 (24 h after seeding cells), cells were exposed to either only RA (10^{-6} M) **(RA)**, to

RA (10^{-6} M) in the presence of 50 μg of *R. caracolito* extract/ml of culture medium **(RA/ext)** or to RA (10^{-6} M) in the presence of 0.05% DMSO **(RA/DMSO)**.

To have a complete set of controls and to monitor the effect of the extract without inducing cells to neuronal differentiation by RA, cells were seeded in three separate culture dishes on day -1 and treated on day 0 (24 h after seeding cells). On that day, one group of cells (culture plate) was exposed to the extract (50 μg/ml of culture medium) **(N1/ext)**, the second group was exposed to 0.05% DMSO **(N1/DMSO)** and the third group to no testing compound **(N1).** All culture dishes were incubated in N1 medium-supplement from day 1 to day 4 prior to harvesting on day 4 of incubation (same time as in differentiating cells). In each set of experiments, one group of P19 EC cells (one culture plate) was seeded at the same time and density as the other testing cultures and harvested 6 days after plating (same time as in other cultures) **(P19 EC cells)**.

An equal total protein concentration (9 μg) of each of the above samples was loaded per lane of SDS-PAGE followed by either staining the gels with Coomassie blue or submitting them to immunoblot experiments. The latter experiments were performed in order to detect reactive bands using TUJ1 antibody. TUJ1 is an antibody with definite specificity for β-III tubulin (Lee *et al.*, 1990_a). The gel electrophoresis shown in figure 10 represents the protein composition of each testing culture. Western blot analysis using TUJ1 antibody revealed a single reactive band at approximately 55 kDa (figure 11). To control the specific immunoreactvity of the antibodies in each set of experiments, one membrane with the same samples as in the testing blot was incubated only with the secondary antibody. In these experiments no immunoreactivity was observed. As depicted in figure 12, densitometric scanning of the membranes did not indicate any

significant difference in the β-III tubulin expression level amongst the cultures exposed to RA whether or not in presence of the extract or its carrier solvent. Ratio of β-III tubulin expression level is relative to control (RA) which is considered as 1. The same results were obtained amongst the cultures incubated in the same conditions in the absence of RA. However, as expected there was a significant difference in the β-III tubulin expression level between the cells exposed and not exposed to RA. The expression of β-III tubulin in **P19 EC cells** which were not exposed to RA or N1 medium-supplement (figure 11) shows that these cells are prone to neuronal differentiation if they proliferate for a long period in proper culture conditions and reach confluency. However, as represented in table 5, β-III tubulin expression level in P19 EC cells treated with RA is significantly higher than in untreated cells. These results also indicate that exposing P19 EC cells to N1 medium-supplement (days 1-4) increases the process of neuronal differentiation even without exposing cells to RA on day 0 (in comparison to the cells untreated with either RA or N1 medium-supplement).

3.3.2 Pattern and Localization of Cytoskeletal Elements

3.3.2.1 Cells Differentiated in N1 medium-supplement

One of the major determinants of neuronal morphology is the microtubule cytoskeleton (reviewed by Gordon-Weeks, 2004). As such, in present experiments the possible effects of *R. caracolito* extract on cytoskletal organization of P19 derived neurons was monitored by direct visualization of two microtubular subunits (α- and β-III tubulin). The experiments were conducted by IF staining of these tubulin monomers in neurons differentiated from P19 EC cells whether or not exposed to *R. caracolito* extract.

Figure 10. Electrophoretic profile of proteins in neurons differentiated from P19 EC cells in presence or absence of *R. caracolito* extract.

9 µg proteins of each sample were loaded per lane. **RA** represents P19 EC cells differentiated to neurons by 10^{-6} M of RA (day 0) followed by incubation in N1 medium-supplement (days 1-4), **RA/ext** represents cells differentiated by RA (10^{-6} M), in presence of *R. caracolito* extract (50µg/ml of culture medium) on day 0 followed by incubation in N1 medium-supplement (days 1-4), **RA/DMSO** represents cells differentiated by RA (10^{-6} M), in presence of 0.05% of DMSO (the same percentage of carrier solvent as in the extract) on day 0 followed by incubation in N1 medium-supplement (days 1-4). **P19** represents undifferentiated P19 EC cells harvested on day 6 after plating cells (same time as differentiating cells). Three other control groups **N1, N1/ext and N1/DMSO** represent cells exposed to nothing, to extract only (50 µg/ml of culture medium) or to DMSO only (0.05%) on day 0 followed by incubation in N1 medium-supplement (days 1-4). In all samples, cells were plated on day -1 and harvested on day 4 of differentiation. standards **(St)** used in this gel are prestained broad range standards consisting of myosin, **198** kDa; β-galactosidase, **115** kDa; bovine serum albumin, **93** kDa; ovalbumin, **49.8** kDa; carbonic anhydrase, **35.8** kDa; soybean trypsin inhibitor, **29.2** kDa; lysozyme, **21.3** kDa; aprotinin, **6.4** kDa. The gel presented in this figure is a representative of three experiments performed independently.

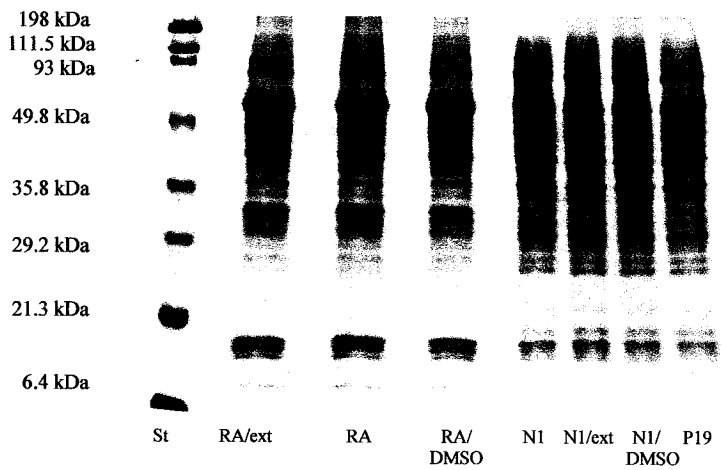

198 kDa
111.5 kDa
93 kDa
49.8 kDa
35.8 kDa
29.2 kDa
21.3 kDa
6.4 kDa
St
RA/ext
RA
RA/ DMSO
N1
N1/ext
N1/ DMSO
P19

Figure 11. Immunoblot labeling of β-III tubulin in neurons differentiated from P19 EC cells in presence or absence of *R. caracolito* extract.

9 μg proteins of each sample were loaded per lane. **RA** represents P19 EC cells differentiated to neurons by 10^{-6} M of RA (day 0) followed by incubation in N1 medium-supplement (days 1-4), **RA/ext** represents cells differentiated by RA (10^{-6} M), in presence of *R. caracolito* extract (50μg/ml of culture medium) on day 0 followed by incubation in N1 medium-supplement (days 1-4), **RA/DMSO** represents cells differentiated by RA(10^{-6} M), in presence of 0.05% of DMSO (the same percentage of carrier solvent as in the extract) on day 0 followed by incubation in N1 medium-supplement (days 1-4). **P19 EC cells** represent undifferentiated cells harvested on day 6 after plating cells (at the same time as differentiating cells). Three other control groups **N1, N1/ext and N1/DMSO** represent cells exposed to nothing, to extract only (50 μg/ml of culture medium) or to DMSO only (0.05%) on day 0 followed by incubation in N1 medium-supplement (days 1-4). In all samples, cells were plated on day -1 and harvested on day 4 of differentiation. standards **(St)** and standards used in this blot are low range biotynylated standards consisting of phosphorylase B, **97.4** kDa; bovine serum albumin, **66.2** kDa; ovalbumin, **45** kDa; carbonic anhyrase, **31** kDa; soybean trypsin inhibitor, **21.5** kDa; lysozyme, **14.4** kDa. The blot presented in this figure is a representative of three experiments performed independently.

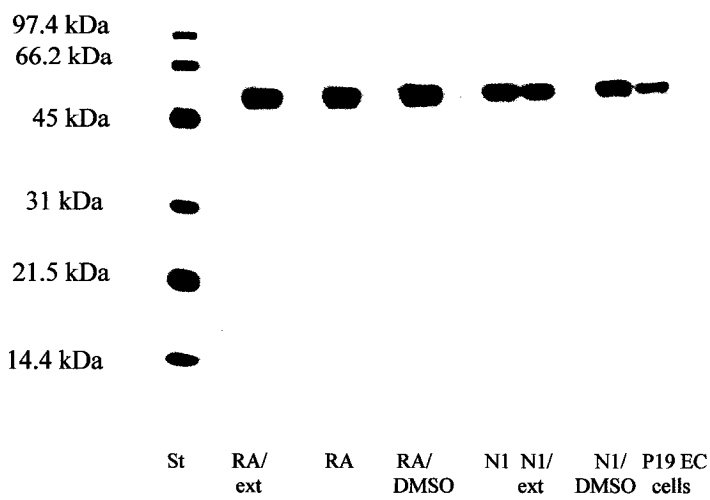
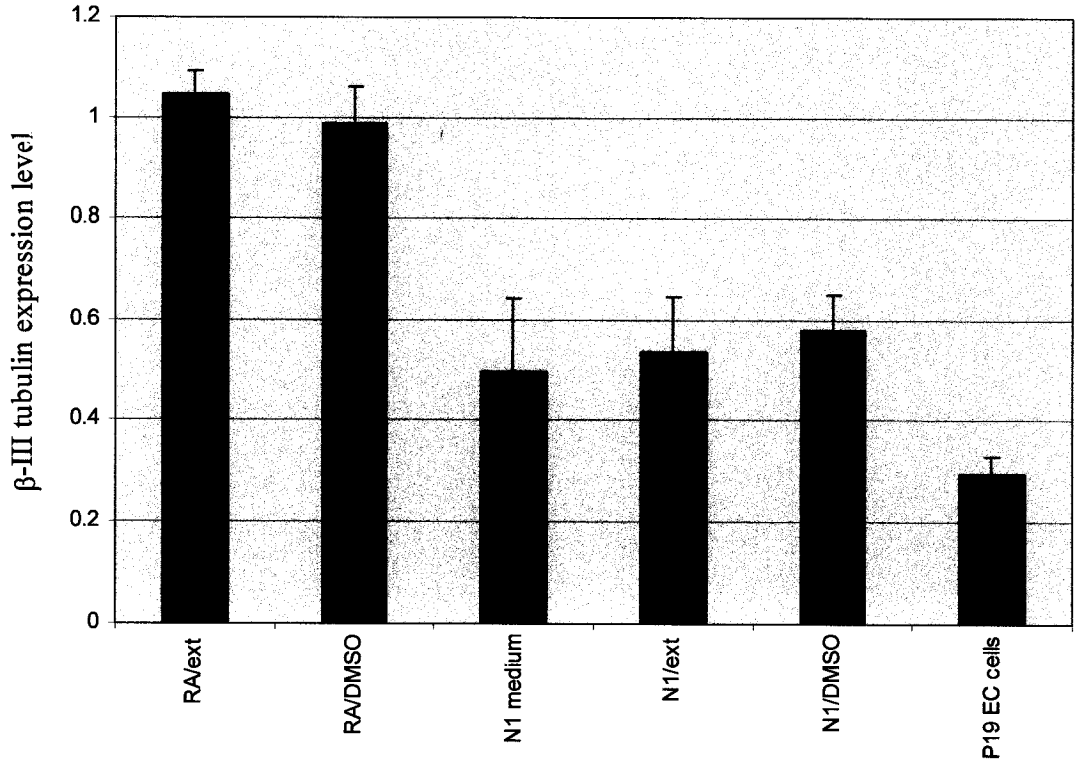

97.4 kDa
66.2 kDa
45 kDa
31 kDa
21.5 kDa
14.4 kDa
St
RA/ ext
RA
RA/ DMSO
N1
N1/ ext
N1/ DMSO
P19 EC cells

Figure 12. Effects of *R. caracolito* extract on β-III tubulin expression level in neurons differentiated from P19 EC cells.

RA represents P19 EC cells differentiated to neurons by 10^{-6} M of RA (day 0) followed by incubation in N1 medium-supplement (days 1-4), **RA/extract (ext)** represents cells differentiated by RA (10^{-6} M), in presence of *R. caracolito* extract (50μg/ml of culture medium) on day 0 followed by incubation in N1 medium-supplement (days 1-4), **RA/DMSO** represents cells differentiated by RA(10^{-6} M), in presence of 0.05% of DMSO (day 0) followed by incubation in N1 medium-supplement (days 1-4). **P19 EC cells** represent undifferentiated cells harvested on day 6 after plating cells (at the same time as differentiating cells). Three other control groups **N1, N1/ext and N1/DMSO** represent cells exposed to nothing, to extract only (50 μg/ml of culture medium) or to DMSO only (0.05%) respectively on day 0 followed by incubation in N1 medium-supplement (days 1-4). In all samples cells were plated on day -1 and harvested on day 4 of differentiation. Each data point represents the average of three independently performed experiments. In each experiment expression level indices were calculated by the immunoreactivity of β-III tubulin band in the testing sample divided by the immunoreactivity of the β-III tubulin band in control (RA). Therefore, ratio of β-III tubulin expression level in control (RA) is considered as 1. Error bars on the graph show the SD among all sets of data (n=3).

Effects of *R. caracolito* extract on β-III tubulin expresion level in neurons differentiated from P19 EC cells



To perform these experiments, neuronal differentiation was induced by exposing P19 EC cells to RA (10^{-6}M) on day 0, in the presence or absence of the extract or its carrier solvent 0.05% DMSO. Differentiation was further supported by incubating cells in N1 medium-supplement from day 1 to day 5 prior to fixing and staining the cells on day 5 of differentiation. Undifferentiated P19 EC cells were also fixed and stained on the same culture day as the other testing populations. The latter experiment was performed in order to assess the cross-reactivity of the antibodies or the positive reaction of β-III tubulin antibody (TUJ1) with undifferentiated P19 EC cells. To control the staining specificity, all cultures were also stained with secondary antibodies only. In such experiments, no staining was observed. Distribution of β-III and α-tubulin in microtubules within developing P19 derived neurons are shown in figures 13-14. As shown in these figures, these tubulin monomers appeared to be incorporated into microtubules within the cell bodies and along the relatively long and thin neurites extended from the predominantly bipolar neurons. Neuronal cell bodies were present in distinct aggregates, with neurites extended outward towards other clusters of neurons. Based on these observations, localization of α- and β-III tubulin in microtubules of P19 derived neurons demonstrated a similar distribution pattern whether or not the cells were exposed to the extract. The signal in undifferentiated cells stained for β-III tubulin was at background level (figure 13).Therefore, as expected undifferentiated cells demonstrated no polymerized β-III tubulin. However, undifferentiated cells stained for α-tubulin exhibited the incorporation of α-tubulin in microtubules polymerized outward in all directions in the cell bodies (figure 14).Based on these observations, undifferentiated P19 EC cells demonstrated the typical centrosome based microtubule arrays of undifferentiated eukaryotic cells.

Moreover, observations of a few random fields of the P19 derived neurons (day 5 of differentiation), stained for β-III tubulin showed that 78%±2.1%, 80.2%±1.8% and 76%±4.2% of the cells exposed to only RA, to RA in presence of the extract and to RA in presence of DMSO were respectively positive for this antigen and so differentiated to neurons. These observations did not reveal any significant change in the proportion of the cells expressing this neuronal marker (β-III tubulin), induced by the extract or its carrier solvent.

These observations also showed that exposing P19 EC cells to the extract results in no significant alteration in the architecture and microtubular organization of P19 derived neurons. Together with the results obtained by densitometric scanning of nitrocellulose membranes immunoblotted for β-III tubulin (described under 3.3.1), they indicate that treatment of P19 EC cells with *R. caracolito* extract does not lead to any significant change in the expression levels, distribution and incorporation of β-III tubulin into P19 derived neuronal processes.

NeuN is a nuclear protein expressed exclusively in neurons (Mullen RJ., *et al.* 1992). As such, expression level of NeuN protein may relate to the degree of neuronal differentiation. To investigate the effect *R. caracolito* extract on neuronal differentiation of P19 EC cells regardless of their neurite outgrowth, the same experiments as mentioned above were performed to probe the expression and distribution of NeuN by IF staining (figure 15). Observations of a few random fields of the cells showed a similar percentage of positive cells for this antigen. These observations showed that amongst the cells exposed to only RA, to RA in presence of the extract and to RA in presence of DMSO, 27%%±2.2%, 28.12%±1.7% and 25.7%±3.1 of cells were positive for NeuN protein,

respectively.

Neun was detected in the nucleus of the neurons and seemed to be detected in the nuclear matrix, nuclear envelope or anywhere in the nucleus. Moreover, this protein was detected in both aggregated and sparse populations of cells. However, as mentioned, only a proportion of cells were visualized as positive for this antigen. Based on these observations, NeuN expression showed a similar localization pattern and distribution in P19 derived neurons whether or not exposed to the extract. Moreover, there was no significant alteration in the number of cells displaying NeuN protein amongst the examined populations of cells.

3.3.2.2 Cells Differentiated in Complex Differentiation Medium

There is mounting evidence showing that cholinergic or GABAergic populations of neurons differentiated from P19 EC cells express functional proteins only in later stages of differentiation (days 6-10) (Parnas and Linial., 1995; McBurney *et al.*, 1988; Fukuchi *et al.*, 1992; Bain *et al.*, 1993; Bain *et al.*, 1994; Staines *et al.*, 1994; Parnas *et al.*, 1998). As such, to compare the functional properties of neurons differentiated from P19 EC cells in the presence or absence of the extract, N1 medium-supplement was replaced with CDM (culturing medium described by Macpherson and McBurney, [1995]). This change in culture conditions of the differentiating cells upon induction by RA provided the neurons with enough nutrients to develop and keep healthy for 10 days in culture. A representative sample of neurons on day 7 of incubation in CDM is shown in figure 16.

To ascertain that incubating P19 cells in CDM (instead of N1 medium-supplement) does not lead to any alteration in the effects of the extract on the

microtubular organization of P19 derived neurons, all sets of experiments described under 3.3.2.1 were repeated. However, N1 medium-supplement was replaced with CDM and cells were incubated in CDM on days 1-10 of differentiation. Differentiating cells were fixed and submitted to IF staining on day 10 of differentiation. Representative pictures of each set of experiments are shown in figures 17-19. The observations were consistent with the results obtained by IF staining of neurons incubated in N1 medium-supplement, indicating that *R. caracolito* extract did not cause any noticeable alteration in the organization of microtubules in neurons differentiated from P19 EC cells. As expected, neuronal cultures on day 10 of differentiation consisted largely of aggregates of neuronal cell bodies with parallel neurites running between them. The more sparsely populated fields are shown in figures 17-19.

3.4 Effects of *Ruptiliocarpon caracolito* Extract on Functional Properties of the Neurons Differentiated from P19 EC Cells

3.4.1 Choline Acetyl Transferase Expression Level

ChAT is a neurotransmitter synthesizing enzyme and the most reliable marker for cholinergic neurons (Nachmansohn and Machado, 1943, reviewed by Dobransky and Rylett, 2003). The following experiments were conducted to assess the possible effects of *R. caracolito* extract on the expression level of the ChAT in P19 derived neurons. ChAT enzyme is a cholinergic marker with a critical function in the synthesis of Ach. As such, expression level of ChAT can be considered as the measure of cholinergic populations and, at least in part, as the functional properties of neurons. To perform these experiments, P19 EC cells were differentiated to neurons in the presence or absence of

Figure 13. Phase contrast and immunofluorescence micrographs of β-III tubulin and DNA in neurons differentiated from P19 EC cells in presence or absence of *R. caracolito* extract (day 5 of differentiation).

Differentiation was induced by **RA** (10^{-6}M) and N1 medium-supplement in presence or absence of *R. caracolito* **extract** (Ext; 50 μg/ml of culture medium) or its carrier solvent (**DMSO**, 0.05%). Cells were fixed and stained on day 5 of differentiation. β-III tubulin is labeled using TUJ1 mouse monoclonal antibody. DNA is labeled by DNA specific dye (Hoechst dye no.33258). Bar, 29μm.

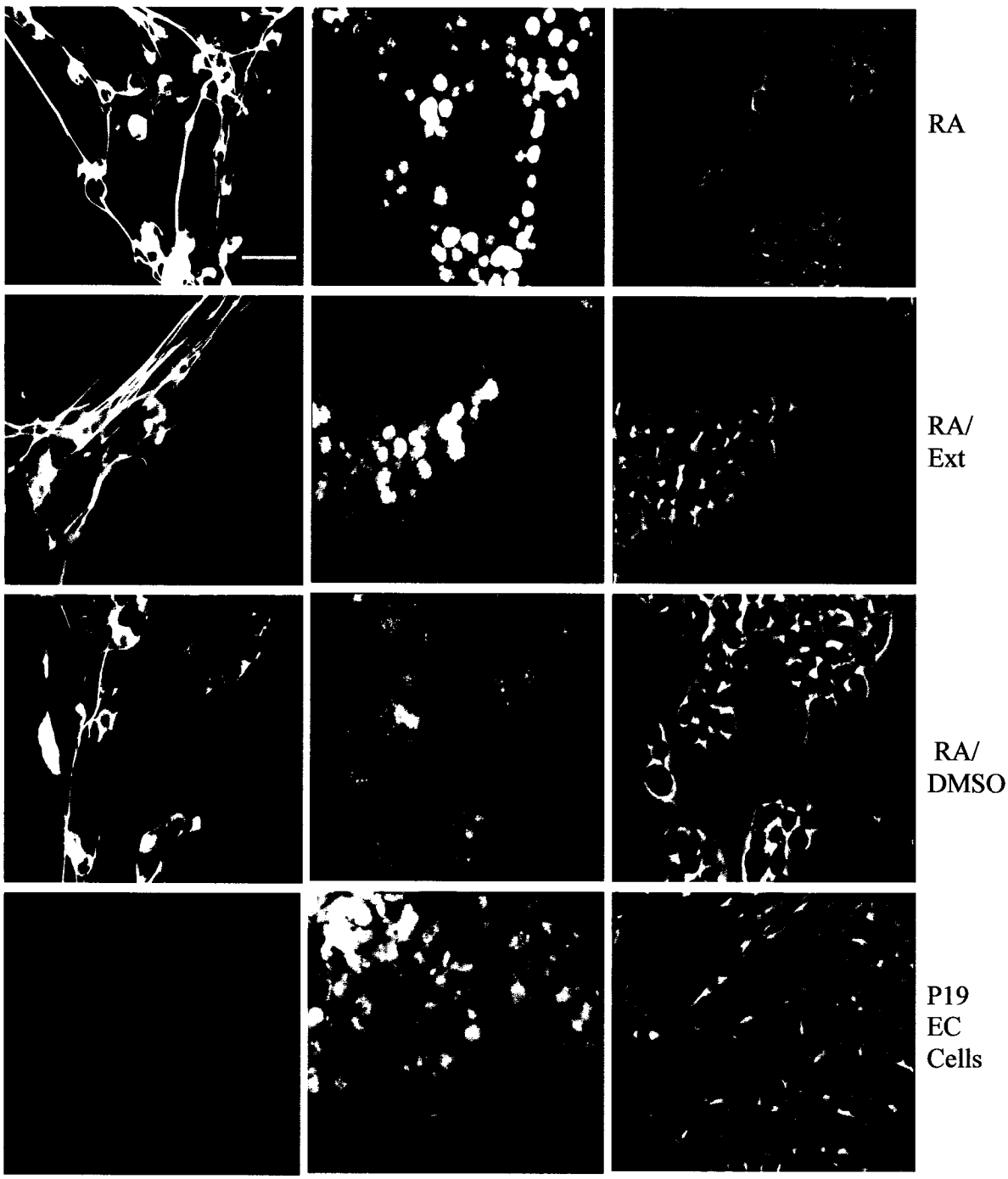

RA
RA/
Ext
RA/
DMSO
P19
EC
Cells
β-III tubulin
DNA
Phase contrast

Figure 14. Phase contrast and immunofluorescence micrographs of α-tubulin and DNA in neurons differentiated from P19 EC cells in presence or absence of *R. caracolito* extract (day 5 of differentiation).

Differentiation was induced by **RA** (10^{-6}M) and N1 medium-supplement in presence or absence of *R. caracolito* **extract** (Ext; 50 μg/ml of culture medium) or its carrier solvent (**DMSO**, 0.05%). α- tubulin is labeled using DM1A mouse monoclonal antibody (IgG). DNA is labeled by DNA specific dye (Hoechst dye no.33258). Cells were fixed and stained on day 5 of differentiation. Bar, 29 μm.

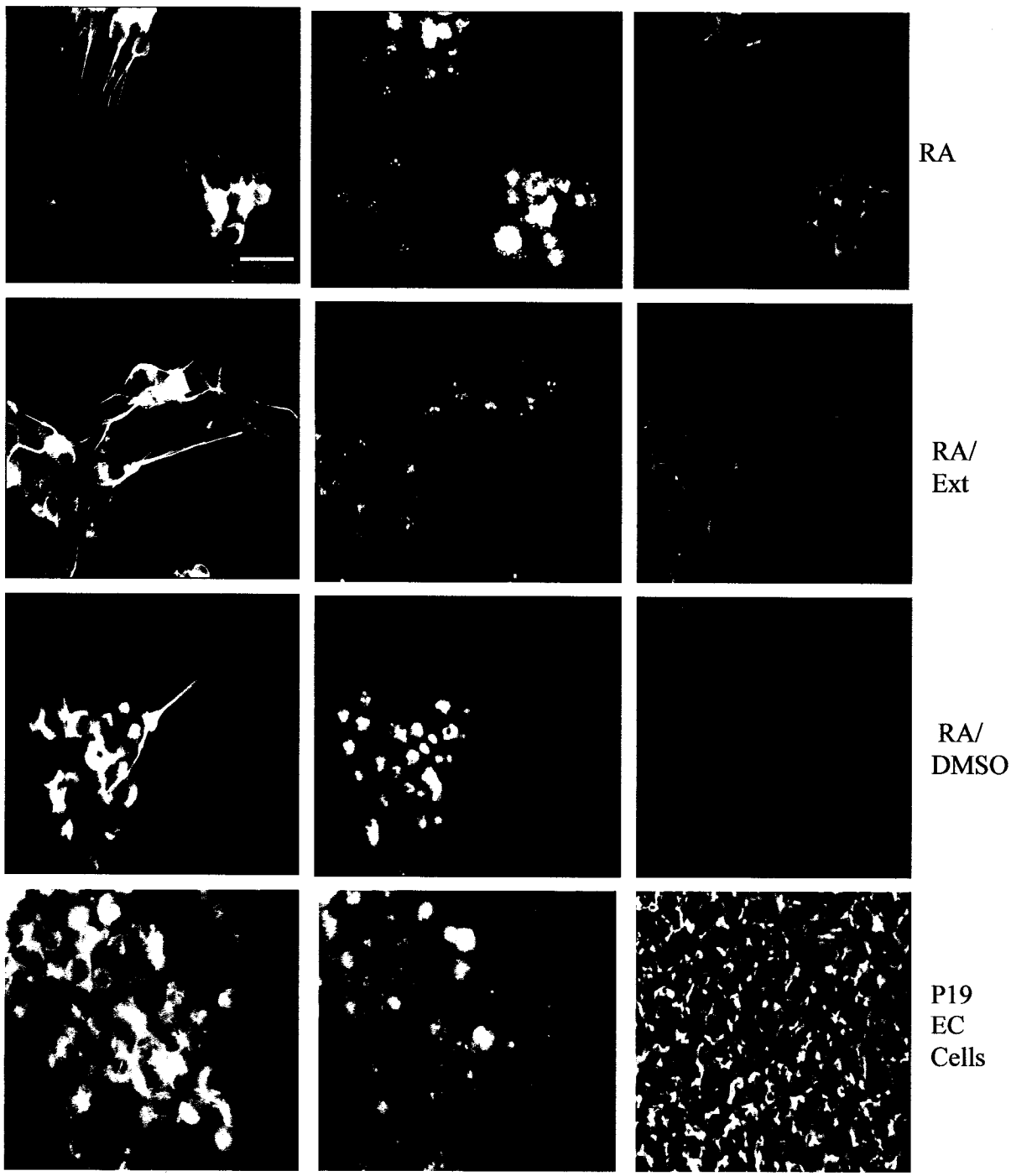

RA
RA/
Ext
RA/
DMSO
P19
EC
Cells
α-tubulin
DNA
Phase contrast

Figure 15. Phase contrast and immunofluorescence micrographs of NeuN and DNA in neurons differentiated from P19 EC cells in presence or absence of *R. caracolito* extract (day 5 of differentiation).

Differentiation was induced by **RA** (10^{-6}M) and N1 medium-supplement in presence or absence of *R. caracolito* **extract** (Ext; 50 μg/ml of culture medium) or its carrier solvent (**DMSO**, 0.05%). Cells were fixed and stained on day 5 of differentiation. NeuN protein is labeled using A60 NeuN mouse monoclonal antibody (IgG). DNA is labeled by DNA specific dye (Hoechst dye no.33258). Bar, 29 μm.

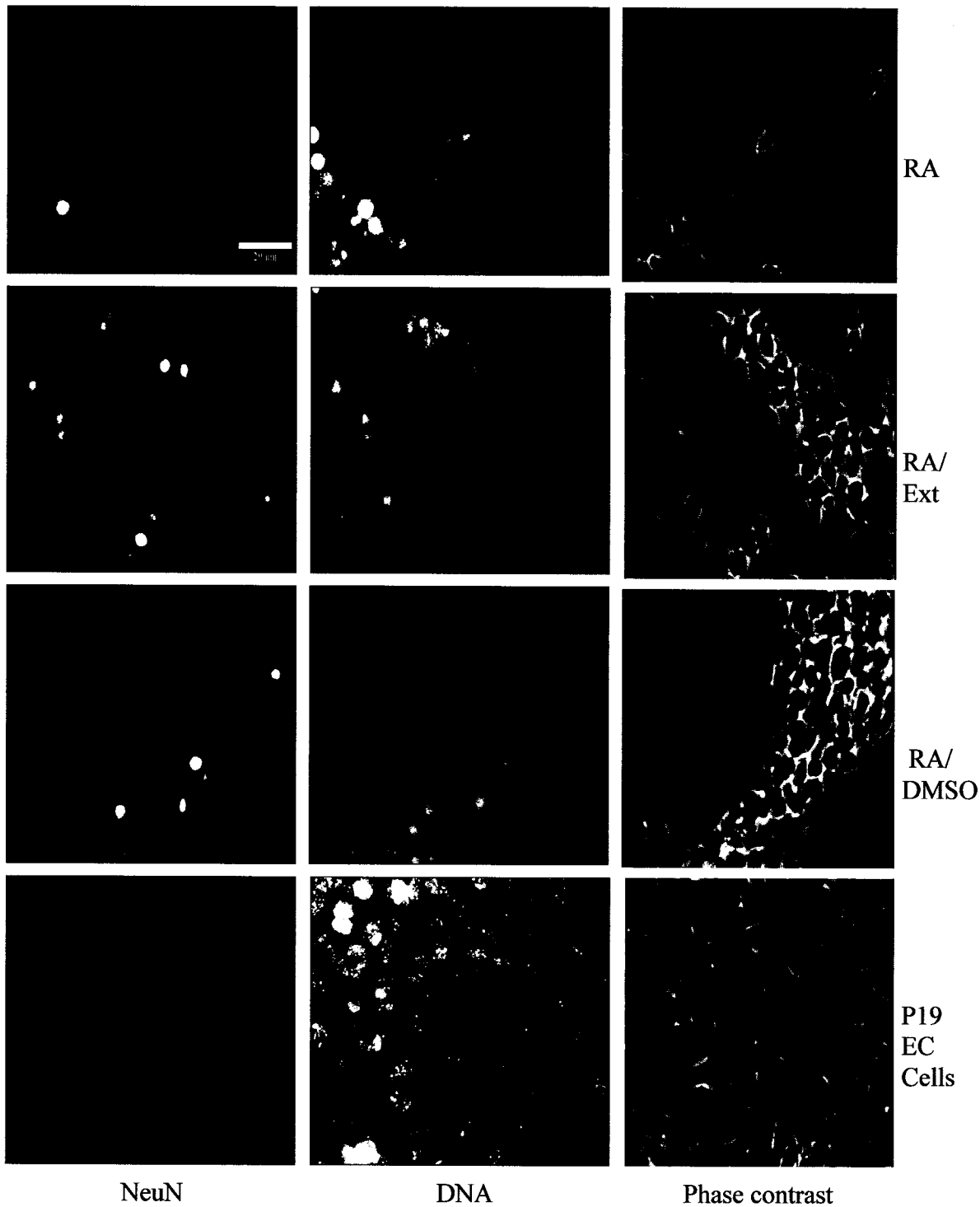
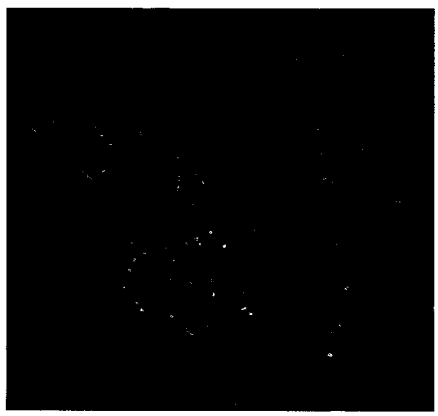
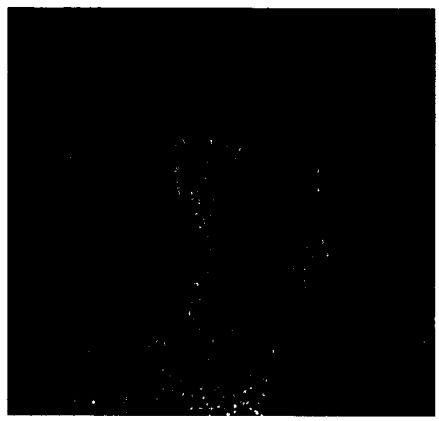

RA
RA/
Ext
RA/
DMSO
P19
EC
Cells
NeuN
DNA
Phase contrast

Figure 16. P19 EC cells differentiated into neurons.

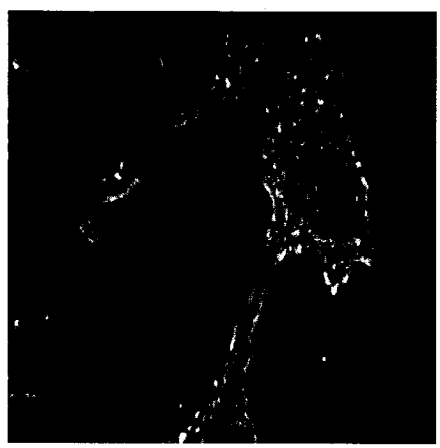
Differentiation was induced by 10^{-6}M of RA (day 0) followed by incubation in complex differentiation medium (CDM; days 1-7) in presence or absence of *R. caracolito* extract (Ext; 50 μg/ml of culture medium) or its carrier solvent (DMSO, 0.05%). Pictures were taken from a 60 mm plate on day 7 of differentiation using an inverted microscope. **RA/CDM** represents cells exposed to RA (10^{-6} M) on day 0 followed by incubation in CDM on days 1-7. **RA/ext/CDM** represents cells exposed to RA (10^{-6} M) in presence of the extract (50 μg/ml of culture medium) on day 0 followed by incubation in CDM on days 1-7. **RA/DMSO/CDM** represents cells exposed to RA (10^{-6} M) in presence of DMSO (0.05%) on day 0 followed by incubation in CDM on days 1-7. Bar, 9μm.



RA/CDM



RA/ext/CDM



RA/DMSO/CDM

Figure 17. Phase contrast and immunofluorescence micrographs of β-III tubulin and DNA in neurons differentiated from P19 EC cells in presence or absence of *R. caracolito* extract (day 10 of differentiation).

Differentiation was induced by **RA** (10^{-6} M) followed by incubation in complex differentiation medium (CDM) in presence or absence of *R. caracolito* **extract** (Ext; 50 μg/ml of culture medium) or its carrier solvent (**DMSO**, 0.05%). Cells were fixed and stained on day 10 of differentiation. β-III tubulin is labeled using mouse monoclonal antibody TUJ1. DNA is labeled by DNA specific dye (Hoechst dye no.33258). Bar, 29μm.

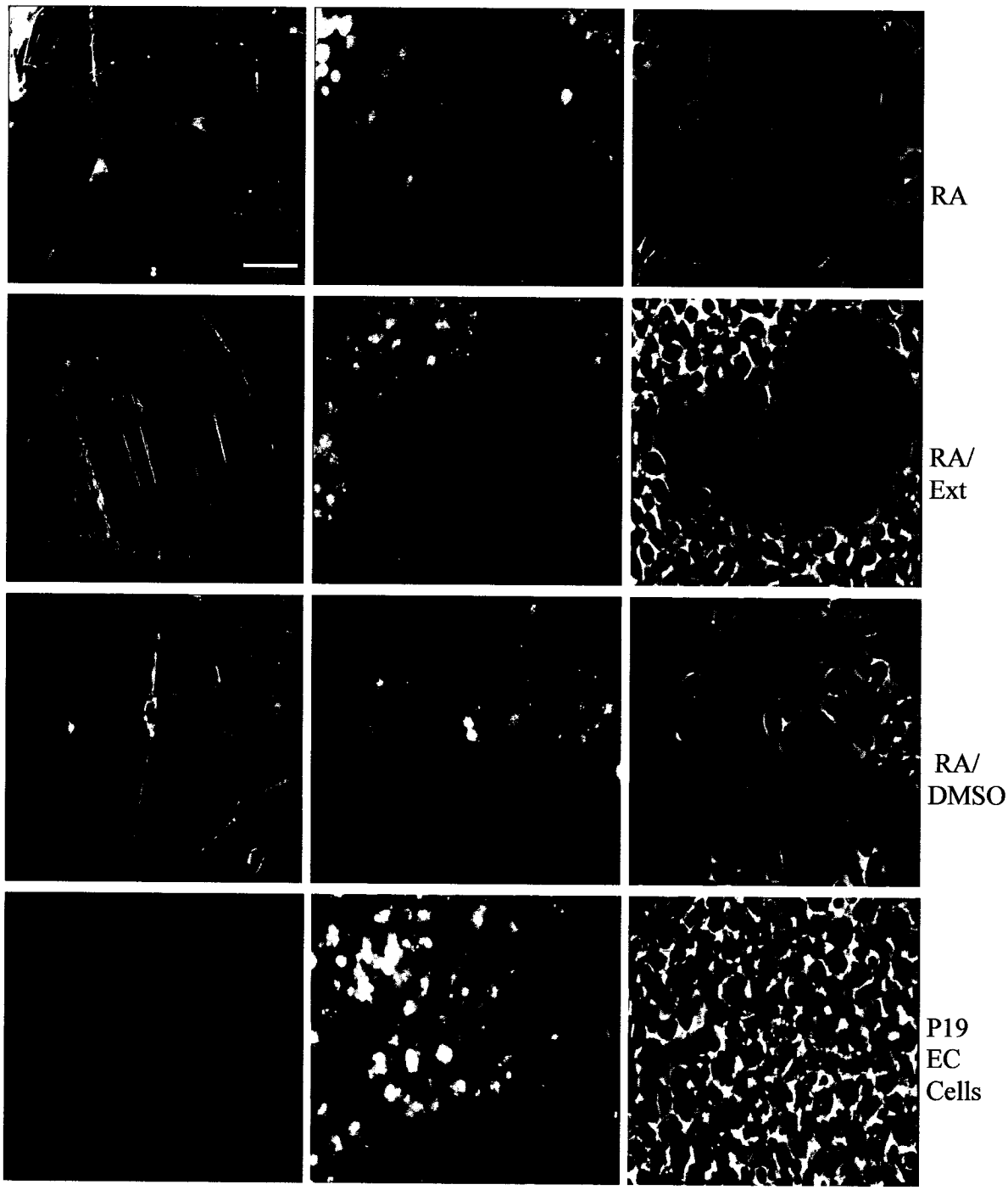

RA
RA/
Ext
RA/
DMSO
P19
EC
Cells
β-III tubulin
DNA
Phase contrast

Figure 18. Phase contrast and immunofluorescence micrographs of α-tubulin and DNA in neurons differentiated from P19 EC cells in presence or absence of *R. caracolito* extract (day 10 of differentiation).

Differentiation was induced by **RA** (10^{-6} M) and followed by incubation in complex differentiation medium (CDM) in presence or absence of *R. caracolito* **extract** (Ext; 50 μg/ml of culture medium) or its carrier solvent (**DMSO**, 0.05%).Cells were fixed and stained on day 10 of differentiation. α-tubulin is labeled using DM1A mouse monoclonal antibody (IgG). DNA is labeled by DNA specific dye (Hoechst dye no.33258). Bar, 69μm.

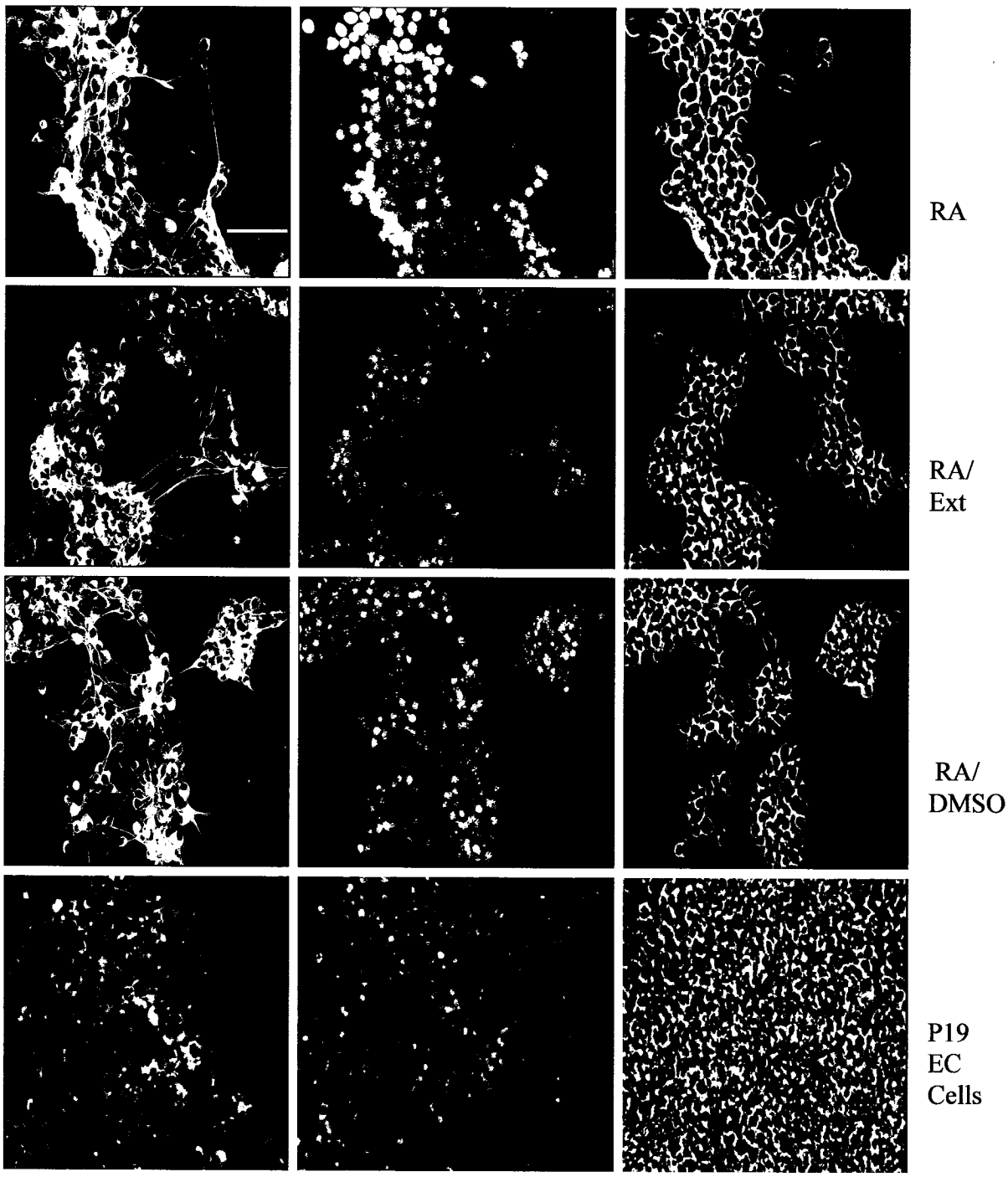
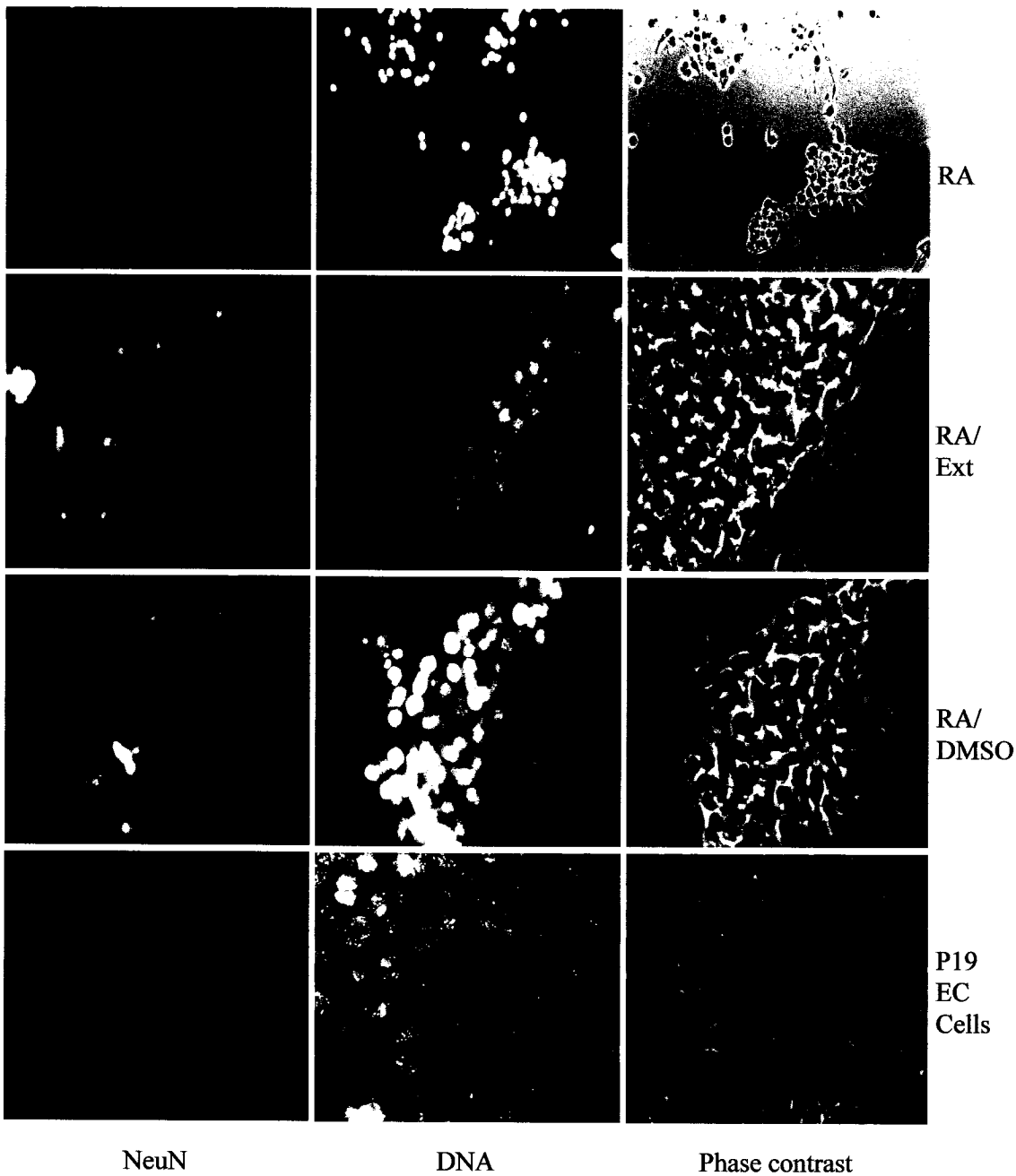

RA
RA/
Ext
RA/
DMSO
P19
EC
Cells
α-tubulin
DNA
Phase contrast

Figure 19. Phase contrast and immunofluorescence micrographs of NeuN and DNA in neurons differentiated from P19 EC cells in presence or absence of *R. caracolito* extract (day 10 of differentiation).

Differentiation was induced **by RA** (10^{-6} M) and followed by incubation in complex differentiation medium (CDM) in presence or absence of *R. caracolito* **extract** (Ext; 50 µg/ml of culture medium) or its carrier solvent (**DMSO**, 0.05%). Cells were fixed and stained on day 10 of differentiation. NeuN protein is labeled using A60 NeuN mouse monoclonal antibody (IgG). DNA is labeled by DNA specific dye (Hoechst dye no.33258). Bar, 29µm.


RA
RA/
Ext
RA/
DMSO
P19
EC
Cells
NeuN
DNA
Phase contrast

the extract or its carrier solvent in three independent experiments as described under 3.3.1. However, in these experiments neurons were incubated in CDM instead of N1 medium-supplement and P19-derived neurons were harvested on day 10 of differentiation. The content of ChAT immunoreactive material was detected by immunoblotting. Figure 20 shows the protein composition of each of the testing cultures. While western blot analysis using ChAT antibody detected no signal in the cells not exposed to RA, three immunoreactive bands were revealed in cells treated with RA and harvested on day 10 of differentiation. These bands displayed MWs of approximately 34-, 64- and 68 kDa (figure 21). Furthermore, there was no specific band observed in the blots probed only with secondary antibodies (controls for staining specificity). As depicted in figures 22-24, densitometric scanning of the detected bands did not reveal any significant difference in the expression level of ChAT isoforms amongst the cultures exposed to RA whether or not in presence of the extract or its carrier solvent. Ratio of ChAT expression level depicted in figures 22-24 are relative to control (RA) which is considered as 1.

3.4.2 Choline Acetyl Transferase Localization

The following experiments were set out to investigate if *R. caracolito* extract may affect the cholinergic phenotype of P19 derived neurons in terms of distribution and sub-cellular localization of ChAT. In order to explore these possibilities, P19 derived neurons were immunofluorescently stained for ChAT on day 10 of differentiation whether or not exposed to the extract or its carrier solvent. Undifferentiated cells were also stained to control the positive reaction or cross-reactivity of ChAT antibody to undifferentiated cells. The signal in undifferentiated cells corresponded to background level. In

Figure 20. Electrophoretic profile of proteins in neurons differentiated from P19 EC cells in presence or absence of *R. caracolito* extract (day 10 of differentiation).

12 μg proteins of each sample were loaded per lane. **RA** represents cells differentiated to neurons by exposing cells to RA (10^{-6}M) on day 0 (one day after seeding cells) followed by incubation in complex differentiation medium (CDM) for days 1-10. **RA/ext** represents cells differentiated by exposing cells to RA (10^{-6}M), in presence of *R. caracolito* extract (50 μg/ml of culture medium) on day 0 followed by incubation in CDM for days 1-10. **RA/DMSO** represents cells differentiated by exposing cells to RA (10^{-6}M), in presence of DMSO (0.05%) on day 0 followed by incubation in CDM for days 1-10. **P19 EC** represents undifferentiated cells harvested on day 6 after plating cells at the same time as differentiating cells. Three other control groups **CDM**, **CDM/ext**, and **CDM/DMSO** represent cells exposed to nothing, extract (only) (50 μg/ml of culture medium) or DMSO (only) (0.05%) on day 0 followed by incubation in CDM for days 1-4. Standards (**St**) used in this gel are prestained broad range standards consisting of myosin, **198** kDa; β-galactosidase, **115** kDa; bovine serum albumin, **93** kDa; ovalbumin, **49.8** kDa; carbonic anhydrase, **35.8** kDa; soybean trypsin inhibitor, **29.2** kDa; lysozyme, **21.3** kDa; aprotinin, **6.4** kDa. The gel presented in this figure is a representative of three experiments performed independently.

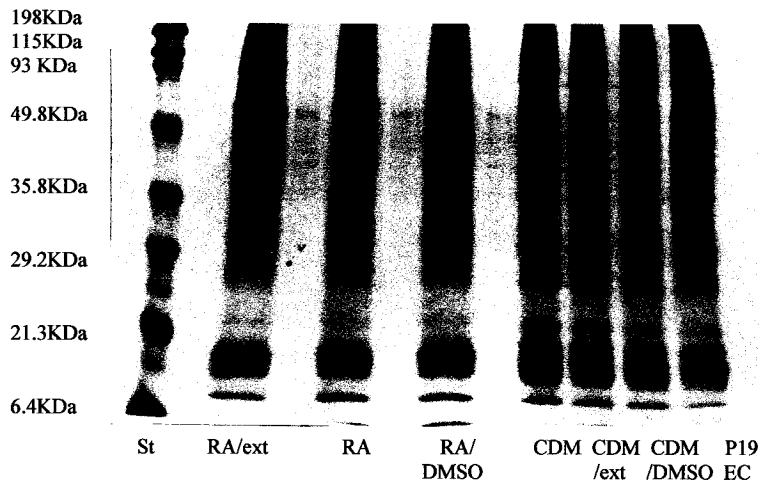
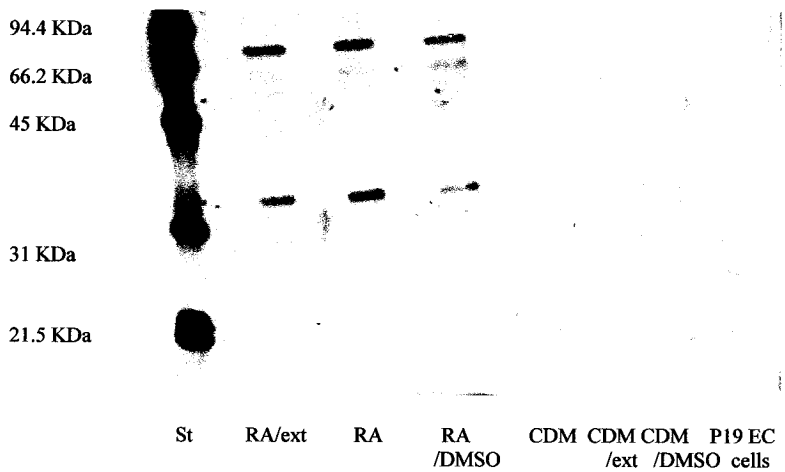

198KDa
115KDa
93 KDa
49.8KDa
35.8KDa
29.2KDa
21.3KDa
6.4KDa
St
RA/ext
RA
RA/ DMSO
CDM
CDM /ext
CDM /DMSO
P19 EC

Figure 21. Immunoblot labeling of ChAT in neurons differentiated from P19 EC cells in presence or absence of *R. caracolito* extract (day 10 of differentiation).

12 μg of each protein homogenates from neurons differentiated from P19 EC cells as well as undifferentiated controls were separated by SDS-PAGE and analyzed by western blotting. **RA** represents cells differentiated by exposing cells to 10^{-6}M of RA on day 0 (one day after plating cells) followed by incubation in complex differentiation medium (CDM) for days 1-10, **RA/ext** represents cells differentiated by exposing cells to RA (10^{-6} M), in presence of *R. caracolito* extract (50 μg/ml of culture medium) on day 0, followed by incubation in CDM for days 1-10, **RA/DMSO** represents cells differentiated by exposing cells to RA (10^{-6} M), in presence of DMSO (0.05%) on day 0 followed by incubation in CDM for days 1-10. **P19 EC** represents undifferentiated P19 EC cells harvested on day 6 after plating cells at the same time as differentiated cultures. Three other control groups **CDM**, **CDM/ext** and **CDM/DMSO** represent cells exposed to nothing, extract (only) (50 μg/ml of culture medium) and just DMSO (only) (0.05%) on day 0 followed by incubation in CDM for days1-4. Bands are detected using ChAT polyclonal antibody. Standards (**St**) used in this blot are low range biotynylated standards consisting of phosphorylase B, **97.4** kDa; bovine serum albumin, **66.2** kDa; ovalbumin, **45** kDa; carbonic anhyrase, **31** kDa; soybean trypsin inhibitor, **21.5** kDa; lysozyme, **14.4** kDa. The blot presented in this figure is representative of three experiments performed independently.


94.4 KDa
66.2 KDa
45 KDa
31 KDa
21.5 KDa
St
RA/ext
RA
RA /DMSO
CDM
CDM /ext
CDM /DMSO
P19 EC cells

Fiure 22. Effect of *R. caracolito* extract on ChAT (~68 kDa) expression level in neurons differentiated from P19 EC cells (day 10 of differentiation).

Neuronal differentiation was induced by exposing cells to RA (10^{-6} M) on day 0 followed by incubation in complex differentiation medium (CDM) on days 1-10. Cell populations were also exposed to different testing compounds on day 0. **RA/ext** represents cells exposed to RA (10^{-6} M) in presence of *R. caracolito* extract (50μg/ml of culture medium) on day 0. **RA** represents cells exposed to RA (10^{-6} M) on day 0. **RA/DMSO** represents cells exposed to RA in presence of DMSO (0.05%) on day 0. No band was detected in four negative control groups of cells which were not exposed to RA. Each data point represents the average of three independently performed experiments. In each experiment, expression level indices were calculated by the immunoreactivity of ChAT band (~68 kDa) in testing sample divided by the immunoreactivity of ChAT band (~68 kDa) in control (RA). Therefore, ratio of ChAT (~68 kDa) expression level in control (RA) was considered as 1. Error bars on the graph show the SD among all sets of data (n=3).

Effect of *R. caracolito* extract on ChAT (~68 kDa) expression level in neurons differentiated from P19 EC cells

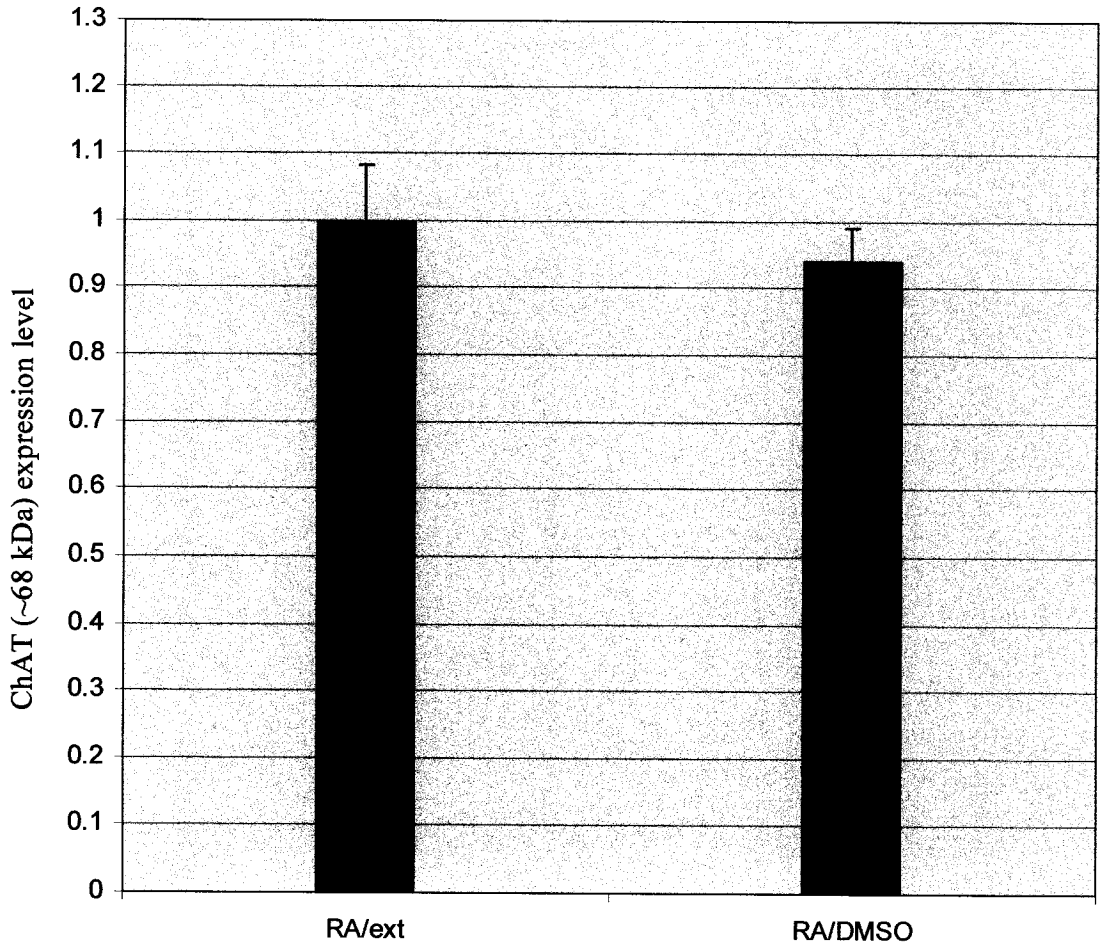


Figure 23. Effect of *R. caracolito* extract on ChAT (~64 kDa) expression level in neurons differentiated from P19 EC cells (day 10 of differentiation).

Neuronal differentiation was induced by exposing cells to RA (10^{-6} M) on day 0 followed by incubation in complex differentiation medium (CDM) on days 1-10. Cell populations were also exposed to different testing compounds on day 0. **RA/ext** represents cells exposed to RA (10^{-6} M) in presence of *R. caracolito* extract (50µg/ml of culture medium) on day 0. **RA** represents cells exposed to RA (10^{-6} M) on day 0. **RA/DMSO** represents cells exposed to RA in presence of DMSO (0.05%) on day 0. No band was detected in four negative control groups of cells which were not exposed to RA. Each data point represents the average of three independently performed experiments. In each experiment, expression level indices were calculated by the immunoreactivity of ChAT band (~64 kDa) in testing sample divided by the immunoreactivity of ChAT band (~64 kDa) in control (RA). Therefore, ratio of ChAT (~64 kDa) expression level in control (RA) was considered as 1. Error bars on the graph show the SD among all sets of data (n=3).

Effect of *R. caracolito* extract on ChAT (~64 kDa) expression level in neurons differentiated from P19 EC cells

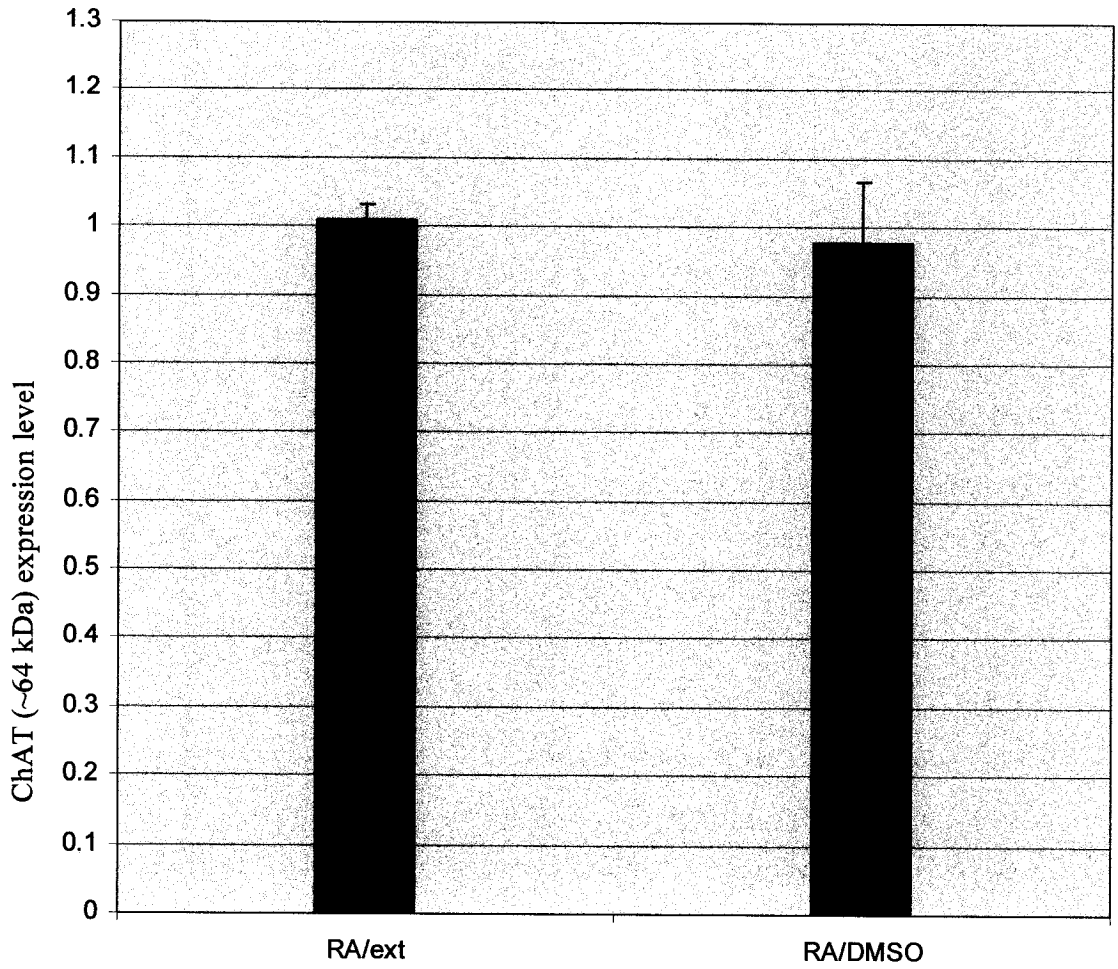


Figure 24. Effect of *R. caracolito* extract on ChAT (~34 kDa) expression level in neurons differentiated from P19 EC cells (day 10 of differentiation).

Neuronal differentiation was induced by exposing cells to RA (10^{-6} M) on day 0 followed by incubation in complex differentiation medium (CDM) on days 1-10. Cell populations were also exposed to different testing compounds on day 0. **RA/ext** represents cells exposed to RA (10^{-6} M) in presence of *R. caracolito* extract (50μg/ml of culture medium) on day 0. **RA** represents cells exposed to RA (10^{-6} M) on day 0. **RA/DMSO** represents cells exposed to RA in presence of DMSO (0.05%) on day 0. No band was detected in four negative control groups of cells which were not exposed to RA. Each data point represents the average of three independently performed experiments. In each experiment, expression level indices were calculated by the immunoreactivity of ChAT band (~34 kDa) in testing sample divided by the immunoreactivity of ChAT band (~34 kDa) in control (RA). Therefore, ratio of ChAT (~34kDa) expression level in control (RA) was considered as 1. Error bars on the graph show the SD among all sets of data (n=3).

Effect of *R. caracolito* extract on ChAT (~34 kDa) expression level in neurons differentiated from P19 EC cells

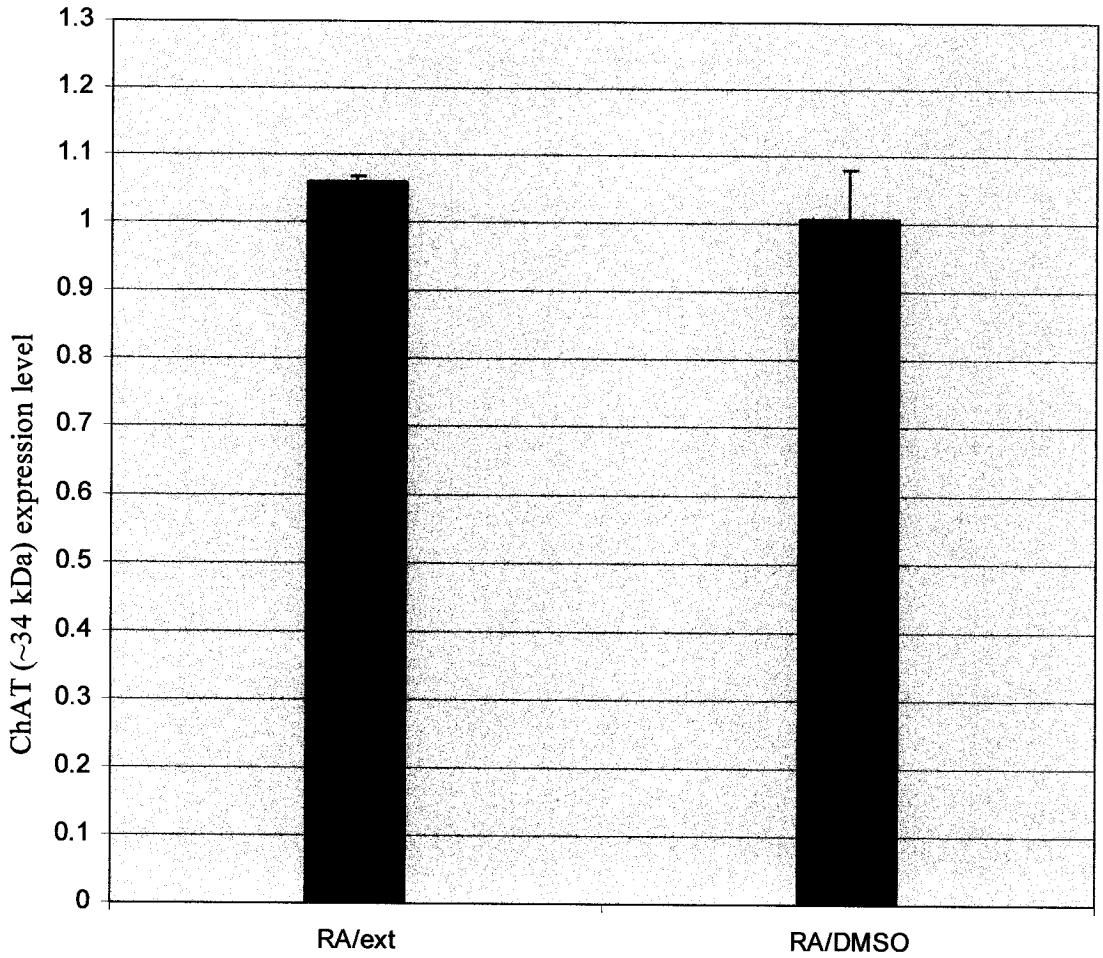
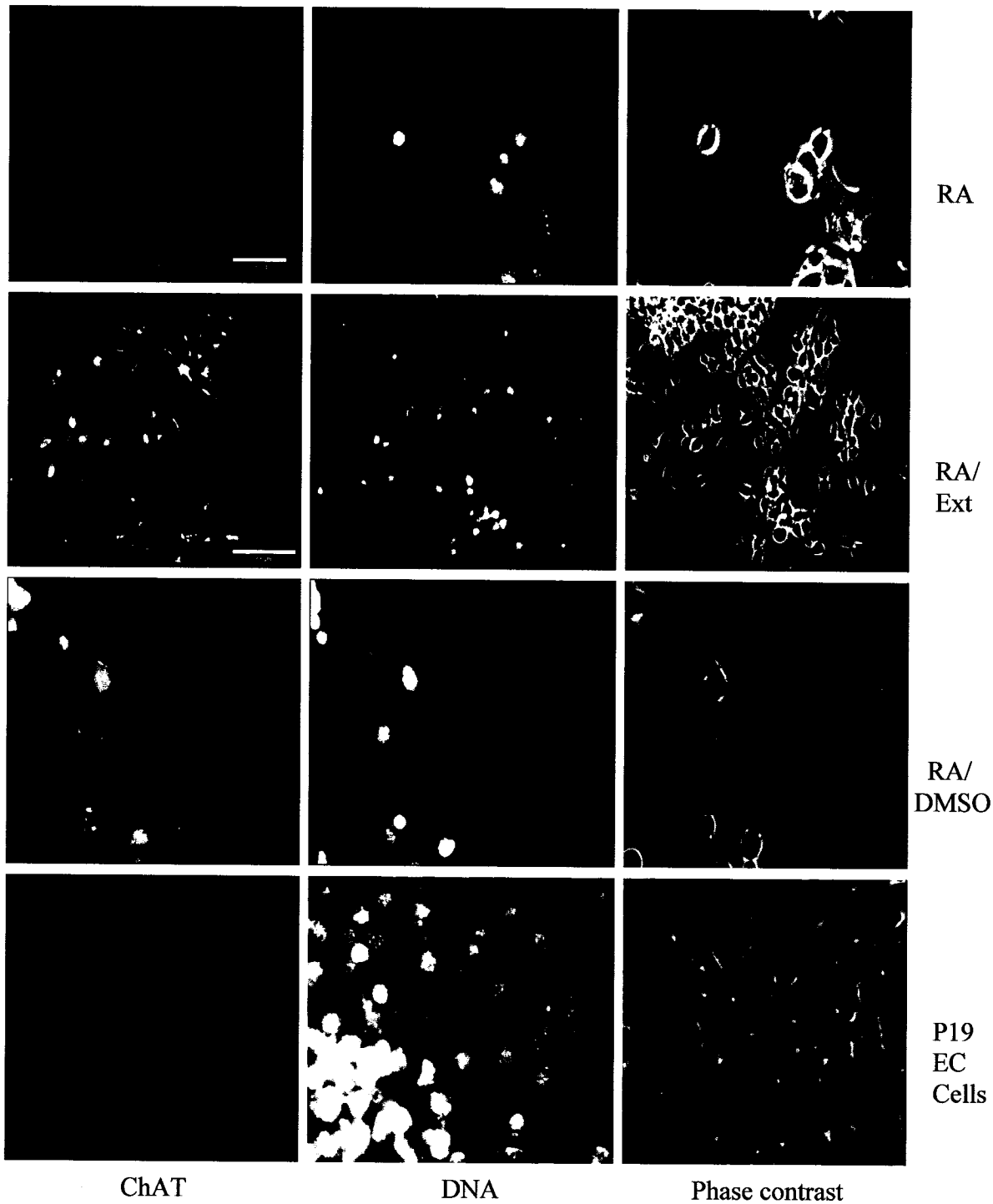


Figure 25. Phase contrast and immunofluorescence micrographs of ChATs and DNA in neurons differentiated from P19 EC cells in presence or absence of *R. caracolito* extract (day 10 of differentiation).

Differentiation was induced by **RA** (10^{-6} M) followed by incubation in complex differentiation medium (CDM) in presence or absence of *R. caracolito* **extract** (Ext; 50 μg/ml of culture medium) or its carrier solvent (**DMSO**, 0.05%). Cells were fixed and stained on day 10 of differentiation. ChAT is labeled using rabbit anti-ChAT polyclonal antibody. DNA is labeled by DNA specific dye (Hoechst dye no.33258). Bar for micrographs related to RA/Ext, 69μm and for the rest of the figure, 29μm.


RA
RA/
Ext
RA/
DMSO
P19
EC
Cells
ChAT
DNA
Phase contrast

accordance with the fluorescence microscopic observations, P19 derived neurons showed a similar localization of ChAT whether or not exposed to the extract or its carrier solvent. Moreover, considering the majority of neurons in each testing group of cells, ChAT exhibited a fairly uniform distribution in the cell body and the neuronal processes of P19 derived neurons (figure 25). In summary, this approach led to the finding that intensity, localization and distribution of ChAT immunoreactivity was not significantly altered by *R. caracolito* extract.

3.5 Further Explorations on the Effects of *Ruptiliocarpon caracolito* Extract on Undifferentiated P19 EC Cells

Based on the results obtained from the present study (section 3.2), treating P19 EC cells with *R. caracolito* extract resulted in the reduction of cell proliferation without increasing cell mortality. Moreover, as described under section 3.3 and 3.4, *R. caracolito* extract allowed the cells to differentiate into neurons upon induction by RA with no significant change in structural and/or functional characteristics of the neurons. The significant anti-proliferative effects of the extract on undifferentiated P19 EC cells indicate that there should be some mechanisms and factors involved in the events happening in the cells treated with the extract. Several cellular pathways from the cell cycle phases to any of the intracellular signals which lead to modification of cell proliferation, may be involved in the growth inhibitory activity of the extract. However, rate of growth usually correlates with the degree of differentiation, and undifferentiated cancerous cells tend to grow faster (Taylor, 1984). As such, the following experiments were undertaken to explore what was occurring at the cellular level to explain the growth

inhibitory effect of the extract using three different markers related to the differentiation status of the cells. Moreover, as these markers are expressed in 3 different locations in the cells including intermediate filaments (Endo-A), cell surface carbohydrates (SSEA1) and nuclear specific intermediate filaments (lamins A/C), probing their expression in the cells exposed to the extract in comparison to untreated controls, can let us explore if *R. caracolito* extract may lead to any alteration in expression and/or localization of these antigens as protein components of different sub-cellular locations. Toward this objective, three independent experiments were performed to probe each of the differentiation markers. In each experiment, expression of the marker was probed whether or not cells were exposed to the extract or its carrier solvent. Each set of experiments was conducted as follows.

An identical number of cells ($1.8x10^5$) were seeded on three 60mm cell culture plates (day 0). Twenty-four hours later (day 1), the medium (D-MEM) of the three plates was changed with either the same medium, the same medium containing 50 μg/ml of extract dissolved in DMSO (Carrier solvent) or the same medium containing 0.05% DMSO (the same percentage of DMSO as in the extract). As described under section 3.2.1, the highest growth reduction effect of the extract elicited on day 2 after treatment (in cells seeded on 60mm plates).Therefore, cells in all cultures were fixed and stained with the following antibodies on day 2 after treatment (day three after plating cells):

1) Troma-1 antibody to label EndoA (Brulet et *al.*, 1980) which is a cytokeratin (component of intermediate filaments), expressed only in cells undergoing differentiation (Takihara *et al.*, 2000);

2) anti-lamin A and C antibody to label lamins A/C which are proteins of nuclear specific intermediate filaments (Holaska *et al.*, 2002) and are expressed in P19 EC cells only if they are fully differentiated (Collard and Raymond 1990);

3) anti stage-specific embryonic antigen1 (SSEA1) antibody (Solter and Knowles 1978) to label SSEA1, a cell surface carbohydrate antigen associated with glycolipids and glycoproteins specific to mouse embryonic cells. Expression of this protein is a marker for undifferentiated murine embryonal carcinoma cells (Knowles *et al.*, 1978; Fenderson *et al.*, 1993; Andrews *et al.*, 1994);

4) MAN human antiserum to label MAN antigens which are non-lamin constituents of the nuclear envelope in vertebrate cells (Paulin-Levasseur *et al.*, 1996).

To have controls in each set of experiments, a population of cells was guided toward the process of differentiation. To prepare this control population of the cells in each set of experiments, $8.3x10^4$ cells were seeded on a 60mm plate containing 5ml of complete D-MEM on day -1. On the following day (day 0), cells were exposed to 10^{-6}M of RA in complete D-MEM. Twenty-four hours later, the medium was changed with D-MEM containing 1% N1 medium-supplement (day 1). Finally, the cells were fixed on day 2 of differentiation at the same time as the undifferentiated P19 EC cells. In two days, these cells were merely guided toward differentiation to neurons, astrocytes and fibroblast-like cells. The partial differentiation of cells in two days was sufficient to achieve positive or negative controls as shown in figures 26 and 27.

As the control for staining specificity in each set of experiments, cultures were stained with the secondary antibody only. In such experiments, no specific staining

was observed. Moreover in each set of experiments, labeling with the MAN human antiserum was used as a positive control to assess the quality of the immunofluorescence staining. In these experiments, cells were consistently stained with the MAN human antiserum.

P19 EC cells are pluripotent and they differentiate to different cell types in culture (McBurney *et al.*, 1982). Cells were grown for three days in culture. As such in each group of cells, whether or not exposed to different testing compounds (50 µg/ml of extract dissolved in DMSO or 0.05% of DMSO only), a small number of cells were differentiating as determined by detection of the differentiation markers. However, there was no significant difference in the ratio of differentiating cells whether or not they were exposed to *R. caracolito* extract or its carrier solvent (DMSO).

3.5.1 Expression of Stage Specific Embryonic Antigen 1 (SSEA1)

Amongst the cultures stained with SSEA1 antibody, most of the cells were positive (undifferentiated) (figure 26), although there were some spots containing cells negative for this antigen. Observations of a few random fields of cells showed a similar percentage of negative cells whether or not cultures were exposed to the extract. In the control group, 6.3%±2.6% of the cells were negative for this antibody. In cultures exposed to the extract (50 µg/ml of culture medium), 9.1%±3% of the cells were negative. In cultures exposed to DMSO (0.05%), 10%±4.4% of the cells were negative. Thus, these observations did not reveal any significant alteration in the percentage of differentiating cells induced by *R. caracolito* extract. Based on the data, most cells remained undifferentiated (positive for SSEA1) after three days in culture whether or

not exposed to the extract. However, about 90% of the cells exposed to RA and fixed on day 2 of differentiation were not expressing SSEA1 protein (figure 26).

3.5.2 Expression of Endo-A Proteins

Amongst the cultures stained with Troma-1, majority of the cells remained unlabeled and were therefore undifferentiated (figure 27). However, there were a few differentiating cells in cultures, whether or not exposed to the extract or its carrier solvent. These cells (positive for the antigen) expressed Endo-A and therefore they had entered into the process of differentiation. Observations of a few random fields showed a similar percentage of cells positive for this antigen whether or not the cultures were exposed to the extract. In the control group, 10.1%±1.7% of the cells were positive for this antibody. In cultures exposed to the extract (50 µg/ml of culture medium), 8.1%±1.5% of the cells were positive. In cultures exposed to DMSO (0.05%), 6.4%±2% of the cells were positive. These observations did not reveal any significant alteration in percentage of differentiating cells induced by *R. caracolito* extract. Nevertheless, immunofluorescence staining of the samples exposed to RA showed that approximately 60% of these cells expressed Endo-A protein and so had undergone the process of differentiation (figure 27).

3.5.3 Expression of lamin A/C Proteins

Lamins A/C were not detected in P19 EC cells whether or not they were exposed to the extract. Moreover, lamins A/C were not detected in the cells exposed to RA and fixed on day 2 of differentiation (figure 28). These observations indicated that the cells exposed to RA did not progress into the late stages of differentiation. To control the

staining procedures and in order to examine staining of the nuclear proteins, MAN antigens were stained in each set of experiments. In these experiments, cells were always stained with MAN human antiserum (figure 28).

Figure 26. Phase contrast and immunofluorescence micrographs of SSEA1 and DNA in P19 EC cells.

Cells were incubated in presence **(Extract)** or absence **(Control)** of *R. caracolito* extract (50 μg/ml of culture medium) or its carrier solvent **(DMSO, 0.05%)**. To have a negative control, one of the cultures was **exposed to RA** (10^{-6} M) in order to guide the cells toward the process of differentiation. Cells were fixed and stained on day 2 after exposure to the extract, DMSO or RA (on day 3 after plating cells). SSEA1 is labeled using SSEA1, a mouse monoclonal antibody (IgM). DNA is labeled by DNA specific dye (Hoechst dye no.33258). Bar, 12μm.

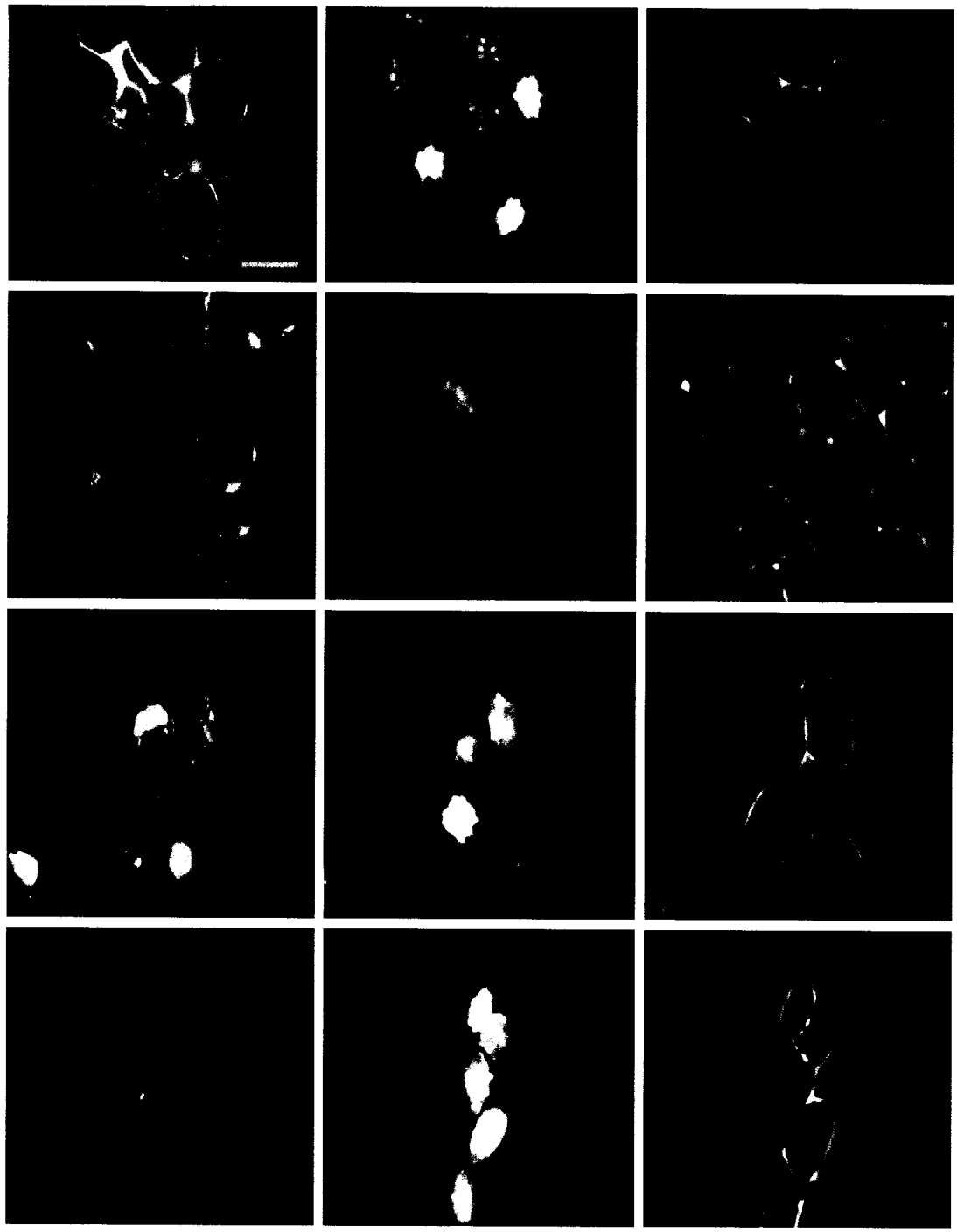

Untreated
Extract
50 µg/ml
DMSO
0.05%
P19 EC
cells,
partly
differentiated
(exposed
to RA)
SSEA1
DNA
Phase contrast

Figure 27. Phase contrast and immunofluorescence micrographs of Endo-A and DNA in P19 EC cells.

Cells were incubated in presence **(Extract)** or absence **(Control)** *of R. caracolito* extract (50 µg/ml of culture medium) or its carrier solvent **(DMSO, 0.05%)**. To have a positive control, one of the cultures was **exposed to RA** (10^{-6} M) in order to guide the cells toward the process of differentiation. Cells were fixed and stained on day 2 after exposing cells to the extract, DMSO or RA (on day 3 after plating cells). Endo-A is labeled using Troma-1, a rat monoclonal antibody (IgG). DNA is labeled by DNA specific dye (Hoechst dye no.33258). Bar, 12µm.

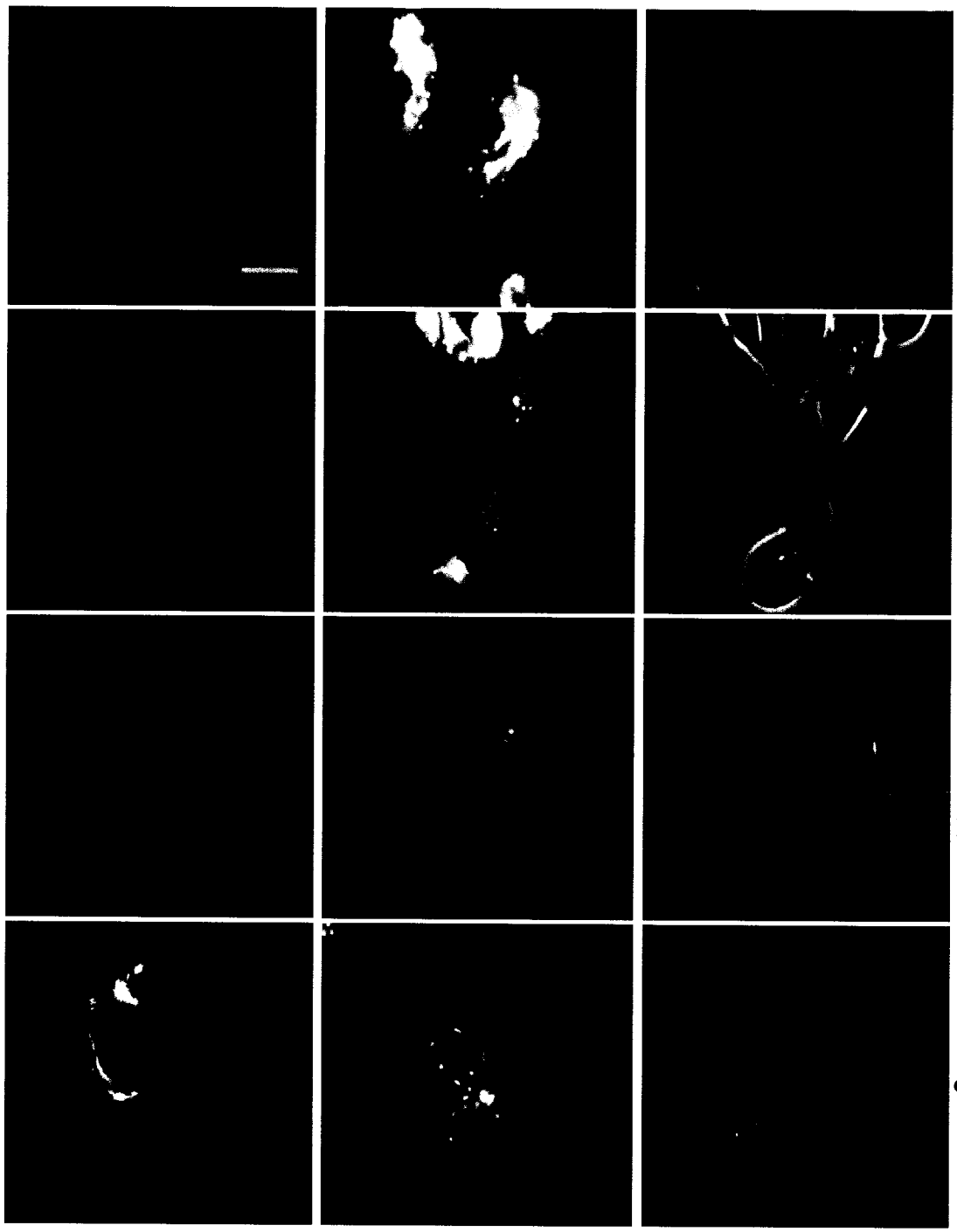
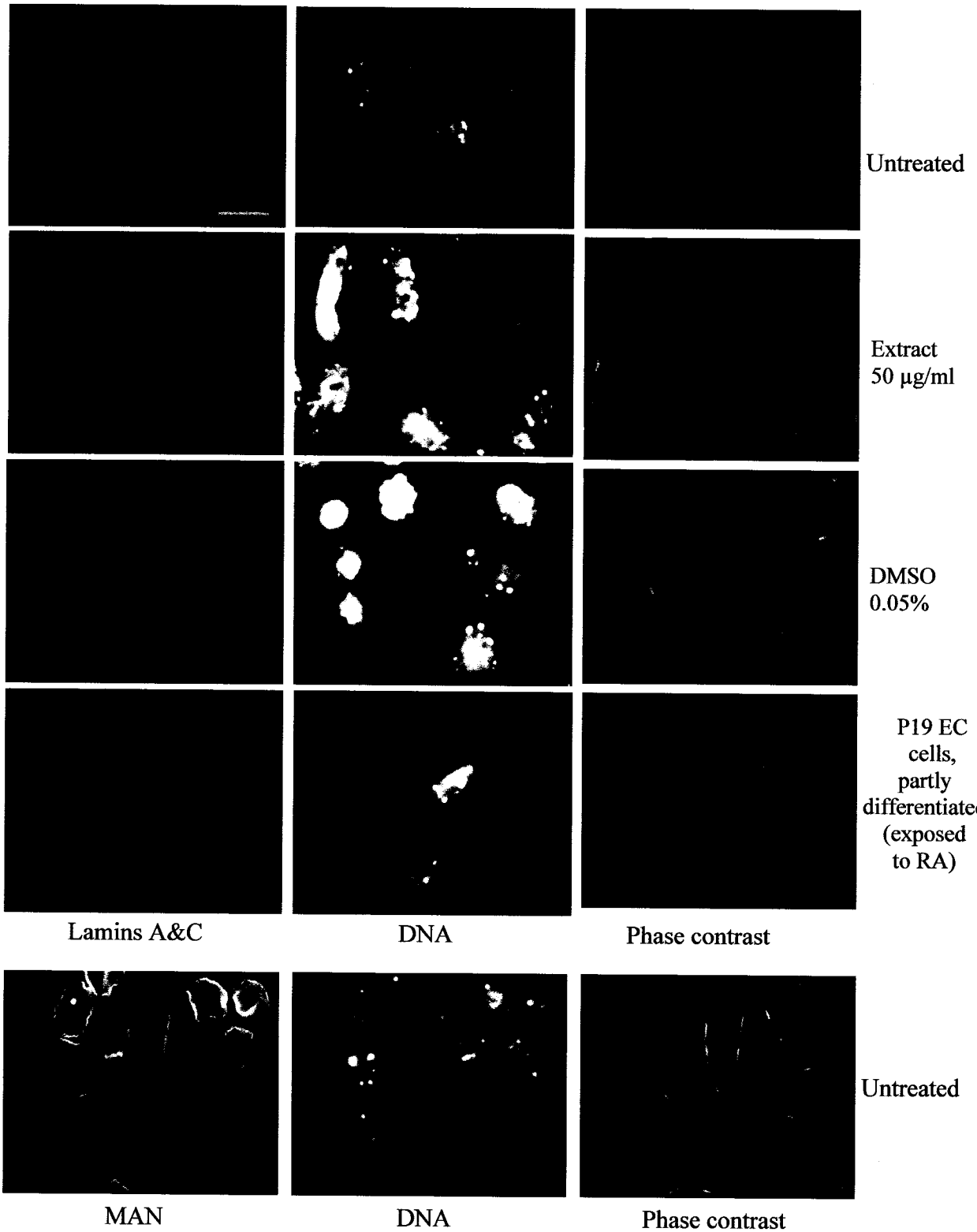

Untreated
Extract
50 µg/ml
DMSO
0.05%
P19 EC
cells,
partly
differentiated
(exposed
to RA)
Endo-A
DNA
Phase contrast

Figure 28. Phase contrast and immunofluorescence micrographs of lamins A and C and DNA in P19 EC cells.

Cells were incubated in presence **(Extract)** or absence **(Control)** of *R. caracolito* extract (50 μg/ml of culture medium) or its carrier solvent **(DMSO, 0.05%)**. One of the cultures was **exposed to RA** (10^{-6} M) in order to guide the cells toward the process of differentiation. Cells were fixed and stained on day 2 after exposing cells to the extract DMSO or RA (on day 3 after plating cells). Lamins A and C are labeled using lamins A and C, a mouse monoclonal antibody (IgG). MAN is stained using the MAN human antiserum. DNA is labeled by DNA specific dye (Hoechst dye no.33258). Bar, 12μm.


Untreated
Extract
50 µg/ml
DMSO
0.05%
P19 EC
cells,
partly
differentiated
(exposed
to RA)
Lamins A&C
DNA
Phase contrast
Untreated
MAN
DNA
Phase contrast

CHAPTER 4

DISCUSSION

4.1 Anti-proliferative Activities of *Ruptiliocarpon caracolito* Extract

Extracts prepared from the bark *R. caracolito* tree have been reported to possess significant anti-proliferative activity against ECB and some strains of fungi. It has also been reported that this extract includes some novel triterpenoids (spirocaracolitones) (MacKinnon, 1995; MacKinnon *et al.*, 1997_a). Triterpenoids are a group of naturally occurring plant compounds that possess anti-proliferative activity on numerous cancer cell lines (reviewed by Setzer and Setzer, 2003). The data presented in this thesis (under section 3.2) first demonstrate the anti-proliferative activity of *R. caracolito* extract against P19 EC cells. P19 is a carcinoma cell line and a good model system to assess the effect of different agents on cell proliferation (Seeley *et al.*, 1998_a; Seeley *et al.*, 1998_b).

Two different methods were used to monitor proliferation of P19 EC cells exposed to the extract in comparison to untreated controls. A relatively good correlation was found between the results obtained from direct cell counting (Trypan blue dye exclusion assay) and the MTT test. Results obtained from both methods revealed a significant anti-proliferative activity of *R. caracolito* extract against P19 EC cells. Viability studies showed that the decrease in cell proliferation was not secondary to the toxicity of the extract. Based on the experimental data collected, a dose- and time-dependent effect was monitored at the concentrations and time intervals tested. The dose-related response was revealed from the fact that no significant adverse effect on cell

growth was observed at the lower doses tested (5-16 μg/ml of culture medium) while the decrease in cell proliferation (induced by the extract) was significant at the higher doses (30-70 μg/ml of culture medium). Moreover, amongst the higher doses tested, the anti-proliferative activity of the extract was slightly enhanced by increasing the concentration of the extract from 30 to 40 μg/ml and finally 50 μg/ml of culture medium. As such, a maximum inhibitory effect was observed at a concentration of 50 μg/ml of culture medium. This effect was reached after 2 days of exposure as determined by trypan blue dye exclusion (39.91%) and 3 days of exposure as determined by MTT assay (32.6%). The slight decrease in anti-proliferative activity of the extract at a concentration of 70 μg/ml , in comparison to the concentration of 50 μg/ml, (table 5) might be due to factors such as the limited solubility of effective substances in the carrier solvent or reaching the maximum effects of active ingredients at concentration of 50 μg/ml. Experiments to characterize the kinetic properties of cell growth inhibition during the 4 culture days of exposure in MTT assay (table 5) showed a time related pattern of growth inhibition. This pattern was revealed from the fact that the anti-proliferative activity of the extract did increase from day 1 to day 3 but decreased following day 3 of exposure. A similar pattern was observed when the direct cell counting method (trypan blue dye exclusion assay) was performed. However, using this method the peak of inhibition was found to be on day 2 following exposure and not on day 3. The increase in anti-proliferative activity could be related to the accumulative effects of the extract. In other words, the proportion of cells affected by the extract might have been increasing by serial addition during the first few days following exposure. The decrease in anti-proliferative effects of the extract after a few days of exposure could be explained by factors such as:(1) reversible growth

inhibition, which might happen following resistance of the cells to the effects of the extract (acquisition of resistance might occur by mechanisms such as the activation of parallel pathways to compensate for the effect(s) and may require a few days to happen) and (2) reduction in the amount of effective compounds of the extract (this reduction might be related to a variety of factors such as degradation or decomposition of effective ingredients after a few culture days).

The anti-proliferative effects of *R. caracolito* extract against P19 EC cells clearly indicate a biologically relevant activity of the compounds present in the extract. However, this effect might be attributed to various (and perhaps yet unidentified) compounds through different biochemical mechanisms. Considering the mounting evidence regarding the anti-proliferative effects of plant derived triterpenoids (reviewed by Setzer and Setzer, 2003), these compounds seem to be attractive candidates for active ingredients of *R. caracolito* extract. However, further investigations are required to characterize the effective substances.

The focus of this part of the study was to determine whether the proliferation of P19 EC cells would be altered in response to *R. caracolito* extract. Biochemical mechanism(s) by which *R. caracolito* extract decreases the proliferation of P19 EC cells remain an intriguing challenge for the future. The experimental data collected over 4 days of exposure revealed that cell proliferation was apparently slowing down, as cells were able to reach confluency with a delay, in comparison to untreated populations of cells. As such, it seems likely that *R. caracolito* extract may exert a cytostatic (inhibitory action on cell growth or cell division) effect on P19 EC cell proliferation. To explore what was occurring at the cellular level to explain the growth inhibitory effect of the extract,

expression of three differentiation markers was detected in P19 EC cells exposed to the extract in comparison to untreated cultures (controls). Microscopic visualization of the markers was conducted on the cells treated with the extract, at the concentration and on the day of exposure that exerted the highest anti-proliferative activity on the cells, in comparison to the untreated cultures (controls). The results obtained from these experiments did not reveal any significant alteration in differentiation status of the cells exerted by the extract. As such, it seems likely that the growth reduction of P19 EC cells in response to *R. caracolito* extract was not related to the differentiation pathways.

4.1.1 Plant-derived Anti-proliferative Agents and Mechanisms of Action

There could be several mechanisms responsible for the anti-proliferative effects of *R. caracolito* extract. However, considering a number of mechanisms by which plant-derived triterpenoids inhibit the proliferation of cancer cells and the data obtained from the current experiments, the possibility of a few mechanisms that may explain the anti-proliferative activity of *R. caracolito* extract are discussed.

One of the triterpenoids isolated from the stem bark of *Dysoxylum cauliflorum* showed anti-proliferative activity against a bronchopulmonary carcinoma cell line. This effect was induced by blocking cells in the G1 phase of the cell cycle and therefore irreversible loss of proliferative potential of the cells, which is generally characteristic of the terminal differentiation. Consequently, the proliferation of these cells was inhibited by the induction of differentiation (Benosman *et al.*, 2000). However, the differentiation status of P19 EC cells was not affected by *R. caracolito* extract. As such, our data do not support the same mechanism of action for anti-proliferative activity of *R. caracolito* extract. However, further investigations are needed to have a clear vision of this issue.

Other investigations demonstrated that pure triterpene acids and mostly β-boswellic acid inhibit DNA synthesis in human leukemia HL-60 cells (Huang *et al.*, 2000). Moreover, some triterpenoids (fomitellic acids A and B and ursolic acid) were found to inhibit both mammalian DNA polymerases α and β and human DNA topoisomerases I and II. As shown in these investigations, these triterpenoids inhibited the activity of the enzymes and did not bind to DNA (Mizushina et al., 1998; Tanaka et al., 1998; Mizushina et al., 2000). Experimental data collected in the present study revealed that *R. caracolito* extract has no cytotoxic effects on P19 EC cells as determined by the number of dead cells in treated cultures in comparison to controls. Indeed, monitoring cell proliferation over 4 days of continuous exposure revealed that treated cells were viable and able to replicate. However, proliferation was significantly reduced in comparison to untreated cultures. These data showed that treated cells were able to progress in the cell cycle. Nevertheless, it does not necessarily rule out the possibility of a reversible inhibitory effect of *R. caracolito* extract on the cell cycle progression, which may just slow the process of cell proliferation. As such, flow cytometric analysis of the cell cycle profile of P19 EC cells exposed to *R. caracolito* extract compared to the controls seems to be an approach to elucidate the mechanisms underlying the anti-proliferative effects of the extract.

There are also other mechanisms responsible for the anti-proliferative effects of plant-derived triterpenoids. These mechanisms include inhibition of ornithine decarboxylase (ODC) activity in mouse hepatoma cells. ODC is an enzyme involved in tumor proliferation. Moreover, experiments on the same cells (mouse hepatoma cells) showed that these triterpenoids increase the activity of quinine reductase (QR), an

enzyme that can inactivate certain carcinogenesis (Bomser *et al.*, 1996). These anti-proliferative activities are mostly exerted on tumor cells, as these enzymes (ODS and QR) are mostly involved in regulation of cancer cell proliferation. To have a better view on the anti-proliferative activity of *R. caracolito* extract, it would be good to look into the effects of the extract on the proliferation of non-tumor cells such as 3T3 cells, which are derived from fibroblasts of disaggregated mouse embryos.

Inhibition of protein tyrosine kinase activity is another not cell cycle-related mechanism reported for growth inhibitory effects of plant derived triterpenoids on tumor cells (Hollosy *et al.*, 2000). Tyrosine kinase activity plays a critical role in the signaling pathways that control cell proliferation (Levitzki, 1996).

In summary, the complexity, variety and accelerating pace of evidence unraveling the mechanisms by which triterpenoids exhibit anti-proliferative activity against carcinoma cells are immediately evident considering the large body of literature in this regard. Altogether, the present piece of discussion shows the challenging quality of the explorations wanted to reveal the mechanism(s) through which *R. caracolito* extract decreases the proliferation of P19 EC cells.

4.2 *Ruptiliocarpon caracolito* Extract and Neuronal Differentiation of P19 EC Cells

P19 EC cells can be induced to differentiate into neurons by RA. This induction results in reduction in their proliferation rate (Jones-Villeneuve *et al.*, 1982). This occurs after a lag period and is mainly attributed to the increased duration of S phase in the cell cycle (Mummery *et al.*, 1987_b). Moreover, this induction of neuronal differentiation

results in structural and functional revolutions in differentiating cells, which occur to generate neuronal phenotype. These neurons are shown to be capable of forming morphologically and functionally mature synapses with electrical properties and of synthesizing and responding to excitatory and inhibitory neurotransmitters (Cheun and Yeh, 1991; McBurney *et al.*, 1988; Magnuson *et al.*, 1995; Finley *et al.*, 1996). There are many specific proteins directly involved in the development and function of the neurons during the process of differentiation. The focus of this part of the study was to determine if *R. caracolito* extract would alter the response of P19 EC cells to RA in terms of the structural and functional properties of the neurons.

4.2.1 Structural Criteria

As pointed out in the introduction, during the neuronal differentiation cells transform their mitotic and cytoplasmic microtubule arrays and undergo extensive rearrangements to generate neuronal processes whose functions include neurite outgrowth and establishment and maintenance of neuronal morphology (reviewed by: Baas, 1997; Kobayashi and Mundel, 1998). Microtubules are formed by polymerization of α- and β- tubulins as tubulin monomers (reviewed by Kirschner, 1978). Class III β-tubulin is the β-tubulin isotype which is exclusively expressed in neurons (Frankfurter *et al.*, 1986). Neuronal differentiation is accompanied by many other major changes in the structure and function of the cells in addition to the alterations in composition and intracellular distribution of microtubules during the process of neurogenesis. Another well-known alteration, which occurs during the process of neurogenesis, is the change in the nuclear structure of the cells throughout differentiation. NeuN is a nuclear protein, which is a marker of neuronal differentiation and is suggested to play important roles in

the development of the nervous system (Mullen *et al.* 1992). Considering all these data, the composition and distribution of microtubules and nuclear components may be used as a structural criterion to evaluate the interfering effects of *R. caracolito* extract on the process of neurogenesis.

In this thesis, the expression level and distribution pattern of class III β-tubulin was monitored in order to investigate the possible effects of *R. caracolito* extract on the neuronal differentiation of P19 EC cells in response to RA and/or on the morphology of the differentiated neurons. Since microtubules and tubulin proteins are necessary for axonal outgrowth, it is conceivable that factors which alter neuronal differentiation and neurite outgrowth of the cells could cause a relevant alteration in the expression level of β-III tubulin as a cytoskeletal protein exclusively expressed in neurons. Western blot and densitometric analysis of the β-III tubulin in neurons differentiated from P19 EC cells (induced by RA) showed no significant alteration in the expression level of this protein in the cells exposed to *R. caracolito* extract at the same time with RA (in comparison to the control populations exposed to only RA). Considering the results obtained from these experiments, it seems that *R. caracolito* extract (under the conditions used) does not significantly interfere with RA-induced neuronal differentiation of P19 EC cells.

Moreover, microscopic visualization of α- and β-III tubulin in neurons differentiated from P19 EC cells did not reveal any noticeable alteration in sub-cellular localization and distribution of these microtubular subunits induced by *R. caracolito* extract in comparison to the control differentiating cells (not exposed to the extract).

In summary, results obtained from the observations (α- and β-III tubulins) and quantitative analysis (β-III tubulin) of cytoskeletal elements in the neurons differentiated

from P19 EC cells did not reveal any significant impairment or increase in development of the neuronal phenotype in response to treatment with *R. caracolito* extract. Taken together, neurons differentiated from P19 EC cells (induced by RA) showed a similar general morphology whether or not exposed to the extract or its carrier solvent

To further evaluate the interfering effect of *R. caracolito* extract upon the process of neurogenesis, expression and distribution of NeuN (a neuron specific nuclear protein) was also examined. These experiments demonstrated no significant alteration in the expression and localization of Neun protein in differentiating P19 EC cells (induced by RA) upon treatment with *R. caracolito* extract, in relation to control differentiating cells (exposed to RA only). Results obtained from these experiments are consistent with the experiments on cytoskeletal elements, indicating no effect of *R. caracolito* extract on neuronal differentiation of P19 EC cells.

4.2.2 Functional Criteria

Identification of neurotransmitter substances in neurons can be a prerequisite for their functional analysis. Moreover, identification of neuron types can be based on the substances they contain. This method is called "chemical coding" in the autonomic nervous system (Costa et al., 1986). ChAT is the neurotransmitter synthesizing enzyme and the most reliable marker for cholinergic neurons (Nachmansohn and Machado, 1943, reviewed by Dobransky and Rylett, 2003). Experiments to examine the functional properties of the neurons differentiated from P19 EC cells revealed no significant alteration in the expression level and distribution of ChAT in the cells differentiated in presence of *R. caracolito* extract. These findings suggest that *R. caracolito* extract does not alter the capability of P19 derived neurons to express ChAT as a functional protein.

There are investigations reporting the transport of ChAT along the axon to the nerve endings by slow axoplasmic transport following synthesis in the perikaryon (Fonnum and Malthe-Sørenssen, 1973; Dziegielewska *et al.*, 1976; reviewed by Oda, 1999). As such, the presence of ChAT all along the axons in the neurons differentiated in presence of the extract (as observed in the present experiments) indicates that *R. caracolito* extract exhibits no apparent detrimental effect on the axonal transportation of ChAT to the nerve endings of the P19 derived neurons, where it can catalyze synthesis of Ach. There are studies reporting the susceptibility of cholinergic neurons to cytotoxic insults (Szutowicz *et al.*, 1996). Results obtained from the present work indicate no deleterious or detrimental effects of *R. caracolito* extract on the differentiation of P19 EC cells into cholinergic neurons in terms of the ChAT expression and distribution. Considering the susceptibility of cholinergic populations of neurons, no harmful effect of the extract on differentiation of the cells to these populations reinforces the results demonstrating no significant effect of *R. caracolito* extract on the neuronal differentiation of P19 EC cells.

Moreover, considering the large body of literature investigating the ChAT isoforms (table 1), this enzyme appears to be presented in different MWs depending on the species. However, investigations do not always report identical MWs for the ChAT isoforms even in the same species. ChAT in mouse brain is reported to be composed of three different isoforms with MWs of 28, 50 and 66 kDa (Bulloch *et al.*, 1994). In the experiments performed in the present studies to evaluate the effects of *R. caracolito* extract on the ChAT expression level, three different ChAT isoforms with molecular weights of about 34-, 64- and 68 kDa were detected in the neurons differentiated from P19 EC cells. Considering evidence showing that ChAT is prone to proteolysis (Hersh *et*

al., 1984), the 64 kDa species observed in the present experiments might represent a proteolyzed form of the 68 kDa isoform. However, ChAT is shown to be present in three different isoforms in several species (table 1).

4.2.3 *Vinca* Alkaloids, *R. caracolito* Extract and Neurotoxicity

Vinca alkaloids are isolated from the extracts of the leaves of *Vinca rosea* and their anti-mitotic activity was discovered in late 1950s (Kamat *et al.*, 1958; Noble *et al.*, 1958; Cutts *et al.*, 1960). These compounds are one of the best-known agents that interfere with microtubule function to exhibit anti-cancer activity (Jordan *et al.*, 1991). There are investigations indicating the decreased expression of class III β-tubulin gene in vincristine resistance neuroblastoma cells (Kavallaris *et al.*, 2000). Moreover, decrease in class III β-tubulin expression counteracts with the depolymerizing agents like *Vinca* alkaloids (reviewed by Burkhart *et al.*, 2001). As such, this isotype of β-tubulin is probably more sensitive to the agents with microtubule destructive activity like *Vinca* alkaloids. Investigations also revealed the neurotoxic side effects of V*inca* alkaloids, which are mostly due to the depolymerization of axonal microtubules and inhibition of the axonal transport (Donoso *et al.*, 1977; Green *et al.*, 1977). In rabbits treated with vincristine, neurons were severely affected by degradation (Muzylak and Maslinska, 1992). Moreover, there are investigations indicating the dose dependent formation of paracrystals, made of spiral helices of one or two protofilaments, in the snails neurons incubated with *Vinca* alkaloids *in vitro* (Muller *et al.*, 1988; Muller *et al.*, 1990). In summary, neurotoxicity is the major side effect of V*inca* alkaloids and neuropathy may lead to dose reduction or cessation of the therapy (Todd *et al.*, 1979; Geldof *et al.*, 1998). In the present studies, simultaneous exposure of the P19 EC cells to *R. caracolito* extract

and RA did not lead to any significant alteration in response of the cells to RA in terms of the structural and functional properties of the differentiated neurons. As such, it seems likely that the use of this extract may not be limited by the occurrence of neurological side effects as in *Vinca* alkaloids. However, further investigations are required to ascertain the validity of this assumption.

4.3 Proliferation and Differentiation Pathways

Differentiation is reported to take place in three stages: reversible arrest of proliferation, acquisition of a differentiated phenotype and terminal maturation of the differentiated cells (Wille and Scott, 1986). Moreover, investigations showed that some differentiation inducers decrease nuclear proliferation (mostly by prolongation of the G1 phase of the cell cycle). Mitochondria are semi-autonomous organelles, which can more than double their content per cell cycle. Continuous increase in the mitochondrial-nuclear ratio can guide the cells toward the process of differentiation. As such, slowing cell cycle progression may provide enough time for cytoplasmic (mitochondrial) growth activities to proceed. These cytoplasmic activities can lead to accumulation of proteins required before intermediary and terminal stages of differentiation (reviewed by von Wangenheim and Peterson, 1998). There is also evidence supporting the simultaneous control of differentiation and proliferation pathways. One of these evidence showed that treating erythroleukemic cells with high doses of γ-irradiation delayed G1 and G2 phases of the cell cycle while synthesis of RNA, protein and mitochondrial DNA were insensitive to this irradiation. These effects and consequences thereof led to continued cytoplasmic growth at the advantage of differentiation (Schwenke *et al.*, 1995; reviewed by: Lane, 1992; von Wangenheim and Peterson, 1998). Moreover, there are a number of

differentiation inducers which are used for non-toxic therapy of leukemia based on the simultaneous control of differentiation and proliferation. For instance, all-trans-retinoic acid and Pomegranate (*Punica granatum*) fruit extracts are used for differentiation-related therapy of acute promyelocytic leukemia (Huang *et al.*, 1988; Chen *et al.*, 1995; Kawaii and Lansky, 2004).

Considering all of the above data from the literature in combination with the fact that P19 EC cells are pluripotent and can differentiate into many cell types in culture (McBurney *et al.*, 1982), I tried to examine whether *R. caracolito* extract results in significant alterations in the differentiation status of P19 EC cells under the conditions it causes a significant reduction in the cell proliferation. To conduct these experiments, expression of three differentiation markers (expressed on the cell surface [SSEA1], intermediate filament [Endo-A] and nuclear-specific intermediate filaments [lamins A/C]) were examined in P19 EC cells following 2 days of continuous exposure to *R. caracolito* extract in comparison to the untreated populations. The experimental data collected from the present work showed that *R. caracolito* extract does not inhibit nor increase the differentiation of P19 EC cells as judged by the morphological criteria using antibodies for the three differentiation markers. These results indicate that despite its anti-proliferative activity on P19 EC cells, *R. caracolito* extract did not exert any significant effect on the process of differentiation under the conditions used in this study. As such, it seems likely that the growth reduction of P19 EC cells in response to the *R. caracolito* extract was not related to the differentiation pathways of these cells.

Considering the results obtained in the present study and the relevant published data, the complexity of mechanisms leading to the anti-proliferative activity of the *R.*

caracolito extract, especially with no significant effect on the differentiation process, is evident. However, there are reports indicating the simultaneous inhibition of differentiation and proliferation in embryonal carcinoma cells (F9 EC cell line) (Piersma *et al.*, 1993). Similarly, there are reports showing alterations (increase or decrease) of neuronal differentiation and neurite outgrowth of PC12 pheochromocytoma cells which did not correlate with any change in the cell proliferation (McFarlane *et al.*, 2000). As such, there is evidence for the absence of a dynamic equilibrium between differentiation and proliferation as observed in the present experiments.

4.4 Future Work

The present study demonstrated the growth inhibitory effects of *R. caracolito* extract in absence of cytotoxicity. However, to design targeting and treatment strategies of the extract, further investigations are required. Additional studies will be essential to gain insight into understanding how *R. caracolito* extract decreases cancer cell proliferation and to optimize its activity:

- Isolating and identifying the structure of anti-proliferative compound(s) in *R. caracolito* extract.
- Discerning the cellular mechanism(s) of anti-proliferative activity of *R. caracolito* compounds.
- Investigating the *in vivo* effects of bioactive compounds in *R. caracolito* extract on tumor cells in model animals.

CONCLUSIONS

Taken together, the present data reinforce the importance of natural products as a

source of anti-proliferative agents, which may have some implications in future cancer therapies. This study has resulted in the following conclusions:

- *R. caracolito* extract exerts anti-proliferative activity against undifferentiated P19 EC cells (at concentrations higher than 30 μg/ml) in the absence of cytotoxicity.
- Under the experimental conditions used in the present study, *R. caracolito* extract lets the cells differentiate into neurons with no significant alterations in tubulin composition/organization or in NeuN detection.
- Under the experimental conditions used in the present study, *R. caracolito* extract induces no significant alteration in the functional properties of the neurons differentiated from P19 EC cells. The functional properties were determined in terms of the expression level and distribution of ChAT as a functional protein expressed in cholinergic populations of neurons.
- Under the experimental conditions used in the present study, *R. caracolito* extract did not significantly affect the differentiation status of P19 EC cells.

APPENDIX

Table 2. Normal proliferation and viability of P19 EC cells over 5 days in culture

Days after Plating Cells	Number of Viable Cells x10^4 (Mean±SD)				Number of Dead Cells x10^4 (Mean±SD)			
	Exp* #1	Exp #2	Exp #3	Average	Exp #1	Exp #2	Exp #3	Average
Day 0	18.000**	18.000	18.000	18.000	0	0	0	0
Day 1	17.116 ± 0.638	16.633 ± 0.550	17.116 ± 0.812	16.955 ± 0.633	0±0	0±0	0±0	0±0
Day 2	48.000 ± 6.622	65.000 ± 12.766	73.000 ± 8.328	62.000 ± 13.225	2.200 ± 0.740	5.866 ± 0.869	3.466 ± 1.021	3.844 ± 1.701
Day 3	222.850 ± 30.385	208.000 ± 29.380	279.000 ± 31.49	236.616 ± 40.680	6.900 ± 1.606	10.466 ± 0.986	16.133 ± 7.678	11.166 ± 5.351
Day 4	399.800 ± 59.513	400.000 ± 65.711	498.000 ± 73.00	432.600 ± 73.913	45.600 ± 7.471	52.500 ± 5.150	41.700 ± 11.296	46.600 ± 8.532
Day 5	480.000 ± 75.984	499.733 ± 84.454	553.33 ± 45.14	511.022 ± 70.160	72.500 ± 3.721	63.000 ± 14.93	84.000 ± 23.027	73.166 ± 15.675

* Exp= experiment.

** Each number in this table represents the total number of cells in a 60mm plate

Table 3. Trypan blue dye exclusion assay: treatment of P19 EC cells with R. caracolito extracts (50 µg/ml of culture medium) and its carrier solvent, DMSO (0.05%). P19 EC cell proliferation was monitored in cells treated with the extract or DMSO in comparison to untreated cultures (Control).

Treatment	Mean+/- SD (Viable cellsx10^4)	n	Control		
			P*	Diff of Means	Significant?
Control (D1)	47.313+/-** 7.408	3	-	-	-
DMSO (0.05%) (D1)	50.750+/- 3.393	3	>0.05	3.438	NS
Extract 50 µg/ml*** (D1)	32.784+/- 2.949	3	<0.05	-14.528	Yes
Control (D2)	204.9172+/- 29.858	3	-	-	-
DMSO (0.05%) (D2)	185.833+/- 46.498	3	>0.05	-19.083	NS
Extract 50 µg/ml (D2)	123.117+/- 12.230	3	<0.05	-81.800	Yes
Control (D3)	497.000+/- 66.421	3	-	-	-
DMSO (0.05%) (D3)	499.667+/- 60.812	3	>0.05	2.667	NS
Extract 50 µg/ml (D3)	362.333+/- 13.204	3	<0.05	-134.667	Yes
Control (D4)	527.082+/- 54.779	3	-	-	-
DMSO (0.05%) (D4)	519.333+/- 40.550	3	>0.05	-7.749	NS
Extract 50µg/ml (D4)	458.333+/- 35.72	3	>0.05	-68.749	NS

Abbreviation: NS, not significant; D, day; Diff of Means, difference of means. D1 represents one day after exposing cells to *R. caracolito* extract and the same for D2-D4. Diff of Means represents mean number of viable cells in treated cultures - mean number of viable cells in controls (untreated cultures)..

* P values are compared to negative controls (untreated cultures).

** Each number in this table represents the total number of cells in 60mm plates

*** 50 µg/ml of culture medium

Table 4. MTT assay: treatment of P19 EC cells with R. caracolito extract (30-70 µg/ml of culture medium) and its carrier solvent, DMSO (0.05%). P19 EC cell proliferation was monitored in cells treated with the extract in comparison to cultures treated with the carrier solvent of the extract (0.05% DMSO).

Treatment (day after treatment)	Mean*+/- SD	n	DMSO (0.05%)		
			P**	Diff of Means***	Significant?
DMSO (0.05%) (D1)	0.992+/- 0.022	3	>0.05	0	NS
Extract 70 µg/ml (D1)	0.934+/- 0.096	3	>0.05	-0.058	NS
Extract 50 µg/ml (D1)	0.906+/- 0.071	3	>0.05	-0.086	NS
Extract 40 µg/ml (D1)	0.914+/- 0.100	3	>0.05	-0.076	NS
Extract 30 µg/ml (D1)	0.944+/- 0.103	3	>0.05	-0.048	NS
DMSO (0.05%) (D2)	1.026+/- 0.039	3	>0.05	0	NS
Extract 70 µg/ml (D2)	0.726+/- 0.068	3	<0.001	-0.3	Yes
Extract 50 µg/ml (D2)	0.738+/- 0.026	3	<0.001	-0.288	Yes
Extract 40 µg/ml (D2)	0.781+/- 0.021	3	<0.001	-0.245	Yes
Extract 30 µg/ml (D2)	0.867+/- 0.019	3	<0.001	-0.159	Yes
DMSO (0.05%) (D3)	1.044+/- 0.016	3	>0.05	0	NS
Extract 70 µg/ml (D3)	0.706+/- 0.042	3	<0.001	-0.338	Yes

Treatment (day after treatment)	Mean*+/- SD	n	DMSO (0.05%)		
			P**	Diff of Means***	Significant?
Extract 50 µg/ml (D3)	0.674+/- 0.019	3	<0.001	-0.37	Yes
Extract 40 µg/ml (D3)	0.701+/- 0.056	3	<0.001	-0.343	Yes
Extract 30 µg/ml (D3)	0.726+/- 0.026	3	<0.001	-0.318	Yes
DMSO (0.05%) (D4)	1.003+/- 0.023	3	>0.05	0	NS
Extract 70 µg/ml (D4)	0.803+/- 0.068	3	0.001	-0.2	Yes
Extract 50 µg/ml (D4)	0.745+/- 0.041	3	<0.001	-0.258	Yes
Extract 40 µg/ml (D4)	0.747+/- 0.047	3	<0.001	-0.256	Yes
Extract 30 µg/ml (D4)	0.802+/- 0.051	3	<0.001	-0.201	Yes

*Mean represents average of OD values in each treated group of cells/average of OD values in negative controls (untreated cells). Therefore, mean value in negative controls is considered as 1. **P values are compared to the carrier solvent controls (cells treated with 0.05% DMSO). ***Diff of Means represents mean of OD values of treated cells - mean of OD values of carrier solvent controls (cells treated with 0.05% DMSO). Abbreviations: NS, not significant; D, day; Diff of Means, difference of means. D1 represents one day after exposing cells to *R. caracolito* extract and the same for D2-D4.

Table 5. MTT assay: growth inhibitory effects of *R. caracolito* extract (30-70 µg/ml) from day 1 to day 4 following treatment.

Days following treatment	Percentage of growth reduction in cells exposed to the extract (in comparison to untreated cells)			
	70 µg/ml*	**50 µg/ml**	**40 µg/ml**	**30 µg/ml**
Day 1	6.6%	9.4%	8.6%	5.6%
Day 2	27.4%	26.2%	21.9%	13.3%
Day 3	29.4%	32.6%	29.9%	27.4%
Day 4	19.7%	25.5%	25.3%	19.8%

* µg/ml of culture medium.

Table 6. Quantitative analysis of β-III tubulin expression in neurons differentiated from P19 EC cells.

Treatment	Mean*+/- SD	n	RA/DMSO		
			P**	Diff of Means	Significant?
RA/ext	1.045+/- 0.048	3	>0.05	0.056	NS
RA/DMSO	0.989+/- 0.075	3	>0.05	0	NS
N1 medium	0.497+/- 0.143	3	<0.001	-0.492	Yes
N1/ext	0.536+/- 0.108	3	<0.001	-0.453	Yes
N1/DMSO	0.577+/- 0.074	3	<0.001	-0.412	Yes
P19 EC cells	0.295+/- 0.035	3	<0.001	-0.694	Yes

* Mean represents the average of expression level indices, calculated by the immunoreactivity of β-III tubulin band in the testing sample divided by the immunoreactivity of the β-III tubulin band in control (RA), in 3 independent experiments. Therefore, ratio of β-III tubulin expression leven in control (RA) is considered as 1.

P values are compared to the carrier solvent control **(RA/DMSO), which represents cells plated on day -1, exposed to RA (10^{-6} M) in presence of 0.05% DMSO on day 0 and incubated in N1 medium-supplement from day 1 to day 4 prior to be harvested on day 4 of differentiation.

P19 EC cells represent undifferentiated cells plated at the same time and density as in other cultures and harvested simultaneously with other testing populations of cells (on day 6 after plating cells). In all other cultures cells were plated on day -1, exposed to different testing compounds on day 0 and incubated in N1 medium-supplement from day 1 to 4 prior to be harvested on day 4. The testing compounds used on day 0 were: (1) RA (10^{-6} M) in presence of *R. caracolito* extract (50μg/ml of culture medium) **(RA/ext)**, (2) RA (10^{-6} M) in presence of DMSO (0.05%) **(RA/DMSO)**, (3) keeping cells as untreated on day 0 **(N1 medium),** (4) extract (50μg/ml of culture medium) in absence of RA **(N1/ext)** and, (5) DMSO (0.05%) in absence of RA **(N1/DMSO)**.

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