Bangladeshi Medicinal Plant Extracts Inhibiting Molecular Interactions between Nuclear Factors and Target DNA Sequences Mimicking NF-kB Binding Sites

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Abstract: Several medicinal plants can be employed to produce extracts exhibiting biological effects. The aim of this work was to verify the ability of extracts derived from different medicinal plants of Bangladesh in interfering with specific DNA-protein interactions. The rationale for this study is based on the observation that alteration of gene transcription represents a very promising approach to control the expression of selected genes and could be obtained using different molecules acting on the interactions between DNA and transcription factors (TFs). We have analysed the antiproliferative activity of extracts from the medicinal plants *Hemidesmus indicus, Polyalthia longifolia, Aphanamixis polystachya, Moringa oleifera, Lagerstroemia speciosa, Paederia foetida, Cassia sophera, Hygrophila auriculata* and *Ocimum sanctum*. Antiproliferative activity was assayed on different human cell lines, including erythroleukemia K562, B-lymphoid Raji, T-lymphoid Jurkat and erythroleukemia HEL cell lines. We employed the electrophoretic mobility shift assay (EMSA) as a suitable technique for the identification of plant extracts altering the binding between transcription factors and the specific DNA elements. We found that low concentrations of *Hemidesmus indicus, Polyalthia longifolia, Moringa oleifera* and *Lagerstroemia speciosa*, and very low concentrations of *Aphanamixis polystachya* extracts inhibit the interactions between nuclear factors and target DNA elements mimicking sequences recognized by the nuclear factor kappaB (NF-κB). On the contrary, high amount of extracts from *Paederia foetida, Cassia sophera, Hygrophila auriculata* or *Ocimum sanctum* were unable to inhibit NF-κB/DNA interactions. Extracts inhibiting both NF-κB binding activity and tumor cell growth might be a source for anti-tumor compounds, while extracts inhibiting NF-κB/DNA interactions with lower effects on cell growth, could be of interest in the search of compounds active in inflammatory diseases, for which inhibition of NF-κB binding activity without toxic effects should be obtained.

Key Words: Medicinal plant, transcription factors, NF-κB, gene expression.

INTRODUCTION

It has been estimated that plants are the most important source of medicinal products for more than 80% of the world's population and especially in developing countries [1]. However, despite the wealth of human experience and folklore concerning the medicinal uses of plants, proper scientific investigation has only been applied to a small fraction of them [1-5]. Nevertheless, natural products present in medicinal plants play a major role in the identification of novel drugs for the treatment of several diseases, including infectious diseases and cancer. For instance, recently discovered drugs from natural products include the powerful anti-tumor drug taxol, which was first isolated from the branches of *Taxus brevifolia* (Taxaceae) [6, 7], the immunosupressants cyclosporine A and FK506 [8, 9], and lovastatin [10].

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As far as molecular targets of medicinal plant compounds, molecules regulating transcription are of interest, since alteration of gene transcription is one of the most important approaches to control the expression of selected genes [11-13] and could be achieved by molecules interfering with the interactions between transcription factors and target DNA elements present within the promoters of those genes whose expression is to be modified. Accordingly, inhibition of DNA-protein interactions could be a very promising strategy that can be applied to the development of antitumor and antiviral therapeutics, as well as intervention in several human pathologies. For instance, DNA-binding drugs are of great interest and object of a wide number of research articles, since our and other research groups demonstrated that DNA-binding drugs inhibit the molecular interactions between DNA and transcription factors, leading to alteration of transcription [14].

In this respect, medicinal plants could be a very important source for molecules inhibiting protein/DNA interactions. For instance, whole extracts from *Uncaria tomentosa* [15], *Isodon japonicus* [16] and *Lonicera*

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japonica [17] were found to inhibit the activity of the nuclear factor kappaB (NF-κB) transcription factor. Starting from these observations, several molecules exhibiting anti NF-kB activity were characterized, including sesquiterpene lactones [18-21], the iridoid glycoside aucubin [22], the kaurane diterpene kambakaurin [23] and the flavonoid quercetin [24]. Several mechanisms of action were reported, including alkylation of cysteine 38 of p65 subunit of NF-kB [18], covalent modification of cysteine 62 of p50 subunit [23], reduction of I-kB degradation [25].

The main issue of the present paper was the screening of Bangladeshi medicinal plants, some of them already analysed with respect to inhibition of cell growth [26-28], for their activity in inhibiting the interactions between nuclear factors from human leukemic K562 cells and a double stranded target oligonucleotide mimicking NF-kB binding sites. Extracts of several medicinal plants were considered, including *Hemidesmus indicus, Polyalthia longifolia, Aphanamixis polystachya, Moringa oleifera, Lagerstroemia speciosa, Paederia foetida, Cassia sophera, Hygrophila auriculata* and *Ocimum sanctum*. This study is intended as a first, small scale, screening of extracts from medicinal plants with respect to activity on protein/DNA interactions. Furthermore, we were interested in determining the relationship between inhibition of protein/DNA interactions and effects on cell growth kinetics.

Among the transcription factors, we started our research studying NF-κB transcription factor because it is known to govern the expression of genes involved in the immune response, embryo or cell lineage development, cell apoptosis, cell cycle progression, oncogenesis, repair and fibrosis processes and inflammation [29, 30]. Accordingly, targeting of NF-κB could lead to the development of new pharmaceutical compounds for innovative treatments of several pathologies [31-33].

For instance, inflammation in asthma is characterized by increased expression of several inflammatory genes, including those encoding cytokines, chemokines, adhesion molecules, inflammatory enzymes and receptors. Blockade of the signal of TNF-α (*Tumor Necrosis Factor alpha*), one of the major cytokines, and of its target transcription factors (AP-1 and NF-κB) may be beneficial for treatments of several pathologies characterized by chronic inflammation [34-37].

Since NF-κB plays a role in oncogenesis [29], all the employed medicinal plant extracts were analyzed also for their antiproliferative activity on cultured tumor cell lines.

RESULTS

Effects of Extracts of Bangladeshi Medicinal Plants on *In Vitro* Proliferation of Human Leukemic K562 Cells

The extracts isolated from *Hemidesmus indicus, Polyalthia longifolia, Aphanamixis polystachya, Moringa oleifera, Lagerstroemia speciosa, Paederia foetida, Cassia sophera, Hygrophila auriculata* and *Ocimum sanctum* were tested as potential inhibitors of *in vitro* proliferation of human erythroleukemic K562 cells. The IC_{50} values (i.e., the concentrations of extracts leading to 50% inhibition of K562 cell growth) were determined in order to compare the activities of the different extracts. In Fig. **1** a representative experiment is shown on the effects of increasing amounts of plant extracts on proliferation of K562 cells. Cells were seeded at the initial concentration of 30,000 cells/ml and then cultured for seven days. The IC_{50} values were calculated after four days, when untreated cells are in the logarithmic phase of the cell growth. The highest antiproliferative activity was found using extracts from *Moringa oleifera* (IC_{50} = 12.55 ± 8.45 μg/ml), *Aphanamyxis polystachya* (IC_{50} = 41.1 ± 0.6 μg/ml) and *Lagerstroemia speciosa* (IC_{50} = 46 ± 2 μg/ml). The other tested plant extracts displayed lower antiproliferative activity (Table **1**)

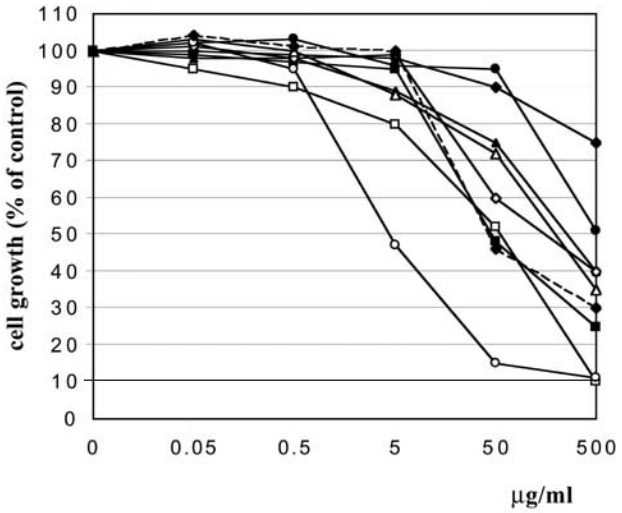

Fig. (1). Effects of extracts from medicinal plants on cell proliferation of K562 cells. K562 cells were seeded at the initial cell concentration of 30,000 cells/ml and then cultured for 7 days in the presence of the indicated amounts of extracts from *Moringa oleifera* (white circles), *Ocimum sanctum* (white squares), *Cassia sophera* (white triangles), *Polyalthia longifolia* (white rhombs), *Hemidesmus indicus* (black circles), *Aphanamixis polystachya* (black squares), *Hygrophila auriculata* (black triangles), *Paederia foetida* (black rhombs) and *Lagerstroemia speciosa* (black rhombs, dotted line).

Effects of Extracts of Bangladeshi Medicinal Plants on *In Vitro* Proliferation of Different Human Tumor Cell Lines

The most active plant extracts derived from *Moringa oleifera, Aphanamyxis polystachya* and *Lagerstroemia speciosa* were tested also on B-lymphoid Raji, T-lymphoid Jurkat and erythroleukemic HEL cell lines. A representative experiment is shown in Fig. **2**, showing the effects of increasing amounts of extracts on these different human tumor cell lines, in comparison with the data obtained using K562 cells. Table **2** shows the IC_{50} values obtained for each cell line. When results reported in Table **1** and Table **2** are considered together, some differences are evident and should be briefly commented. We can observe that the extract of *Moringa oleifera* exhibits the highest antiproliferative activity on K562 and Raji cell lines (IC_{50} = 12.55 ± 8.45 and 1.82 ± 0.23 μg/ml, respectively), but is active also on Jurkat

Table 1. Antiproliferative Activity of Plant Extracts on K562 Cells

Medicinal plant	IC_{50} (μg/ml)
Hemidesmus indicus	550.5 ± 6.5
Polyalthia longifolia	206 ± 4.0
Aphanamixis polystachya	41.1 ± 0.6
Moringa oleifera	12.55 ± 8.45
Lagerstroemia speciosa	46 ± 2.2
Paederia foetida	838 ± 6.0
Cassia sophera	383 ± 5.0
Hygrophila auriculata	526 ± 50
Ocimum sanctum	63.5 ± 5.5

Cell number/ml values were determined after 4 days treatment. Data represent the average ± SD from four different experiments.

and HEL cells, despite at lower level (IC_{50} values being 18 ± 2.0 and 26.8 ± 9.2 μg/ml, respectively). By contrast, the antiproliferative activity of extracts from *Aphanamyxis polystachya* and *Lagerstroemia speciosa* is evident mostly on the Raji cell line (IC_{50} = 3 ± 1.2 and 2.65 ± 0.35 μg/ml, respectively), the same extracts being active also on K562 (IC_{50} = 41.1 ± 0.6 and 46 ± 2 μg/ml, respectively) cell lines despite to a lower extent. On the contrary, the same extracts exhibit lower antiproliferative activity on Jurkat and HEL cell lines.

Plant Extracts and Inhibition of Protein-DNA Interactions

In an effort to determine whether inhibitory activities against transcription factors/DNA interactions are present in extracts from medicinal plants, the same plant extracts that were tested for antiproliferative activity were assayed for their ability in inhibiting the interactions between nuclear factors isolated from the human leukemic K562 cells and a double stranded target oligonucleotide mimicking the binding sites of the transcription factor NF-kappaB.

The results obtained are shown in Fig. **3**. The electrophoretic mobility shift assay was performed using [γ-^{32}P] 5'-end-labelled oligonucleotides. Nuclear extracts were prepared from human leukemic K562 cells. The 5'-labelled oligonucleotides were incubated with plant extracts for 15 min and then nuclear extracts were added. After 15 min binding at room temperature, the samples were electrophoresed at constant voltage (200 V) under low ionic strength conditions (0.25 x TBE buffer = 22 mM Tris-borate, 0.4 mM EDTA) on 6 % polyacrylamide gels. Gels were

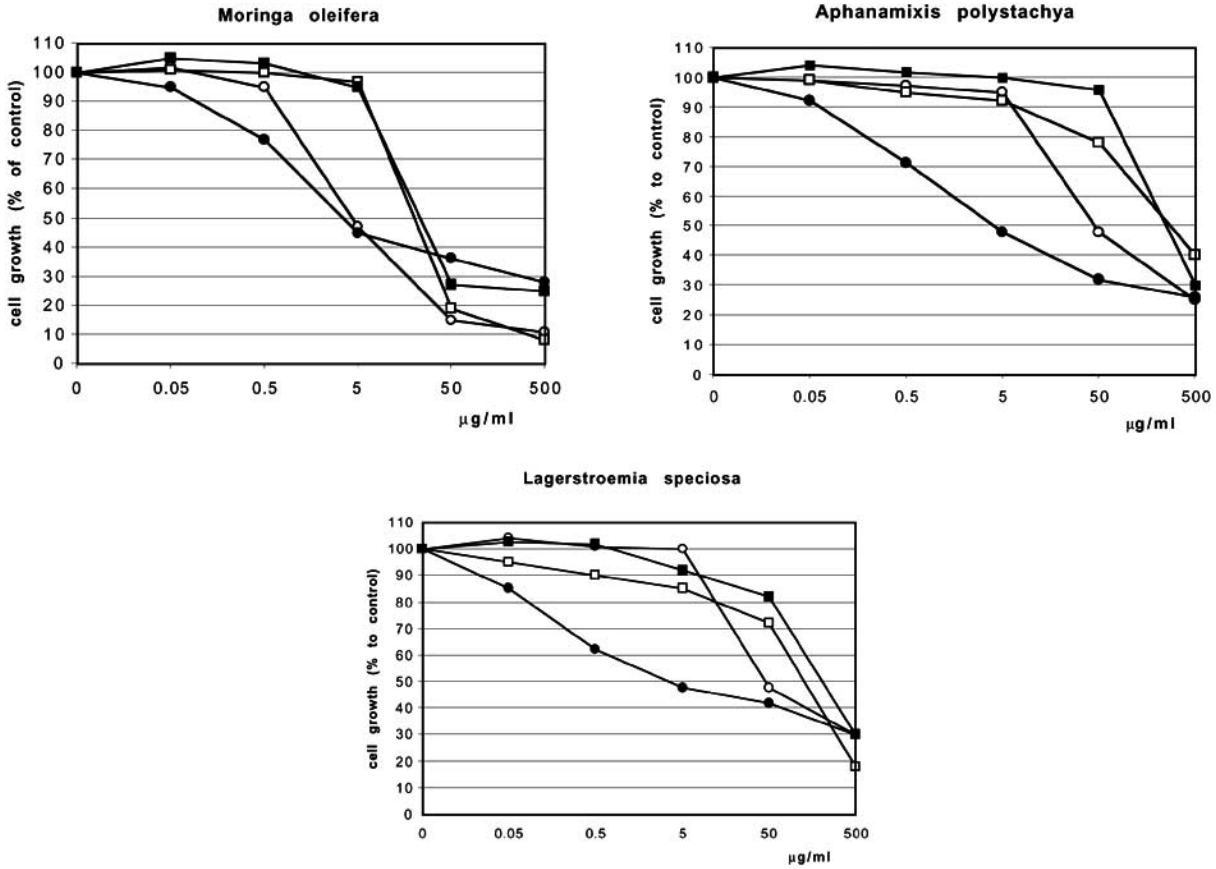

Fig. (2). Inhibitory effects of extracts from *Moringa oleifera*, *Aphanamixis polystachya* and *Lagerstroemia speciosa* on cell proliferation of K562 (white circles), Raji (black circles), Jurkat (white squares) and HEL (black squares) cells. Cells were seeded at the initial cell concentration of 30,000 cells/ml and then cultured for 7 days in the presence of the indicated amounts of extracts.

Table 2. Antiproliferative Activity (IC_{50}, μg/ml) of Extracts of *Moringa oleifera, Aphanamixis polystachya* and *Lagerstroemia speciosa* on Raji, Jurkat and HEL Cell Lines

Medicinal plant	Raji	Jurkat	HEL
Moringa oleifera	1.82 ± 0.23	18 ± 2.0	26.8 ± 9.2
Aphanamixis polystachya	3 ± 1.2	230 ± 5.0	355.8 ± 4,2
Lagerstroemia speciosa	2.65 ± 0.35	185.5 ± 47.5	326.8 ± 15

dried and subjected to standard autoradiographic procedures. Controls with the different concentrations of solvents used to dilute the extracts (water, ethanol, methanol and dimethyl-sulfoxide) were run to exclude effects of them on NF-kB/DNA interactions. The final concentrations of these solvents in the 20 μl EMSA incubation mixture for the highly concentrated samples (100 μg/reaction) were in the range between 0.9 μl and 4.8 μl, depending on the solubility of each sample, and did not interfere with NF-kB/DNA interactions.

The results obtained show that low concentrations of *Lagerstroemia speciosa* (25 μg/binding reaction), *Hemidesmus indicus* (12.5 μg/binding reaction), *Polyalthia longifolia* (12.5 μg/binding reaction) and very low concentrations of *Aphanamixis polystachya* (0.78 μg/binding reaction) extracts inhibit NF-κB/DNA interactions. On the contrary, 100 μg/binding reaction of extracts from *Cassia sophera, Paederia foetida, Hygrophila auriculata* and *Ocimum sanctum* were still unable to inhibit NF-κB/DNA interactions. This was reproducibly observed in three independent experiments.

In Table **3**, the IC_{50} values (concentration leading 50% inhibition of NF-kB/DNA interactions) of the electrophoretic mobility shift assays are reported. When data from Table **I**, Table **II** and Table **III** are compared, it is evident that most of the extracts displaying high antiproliferative activity

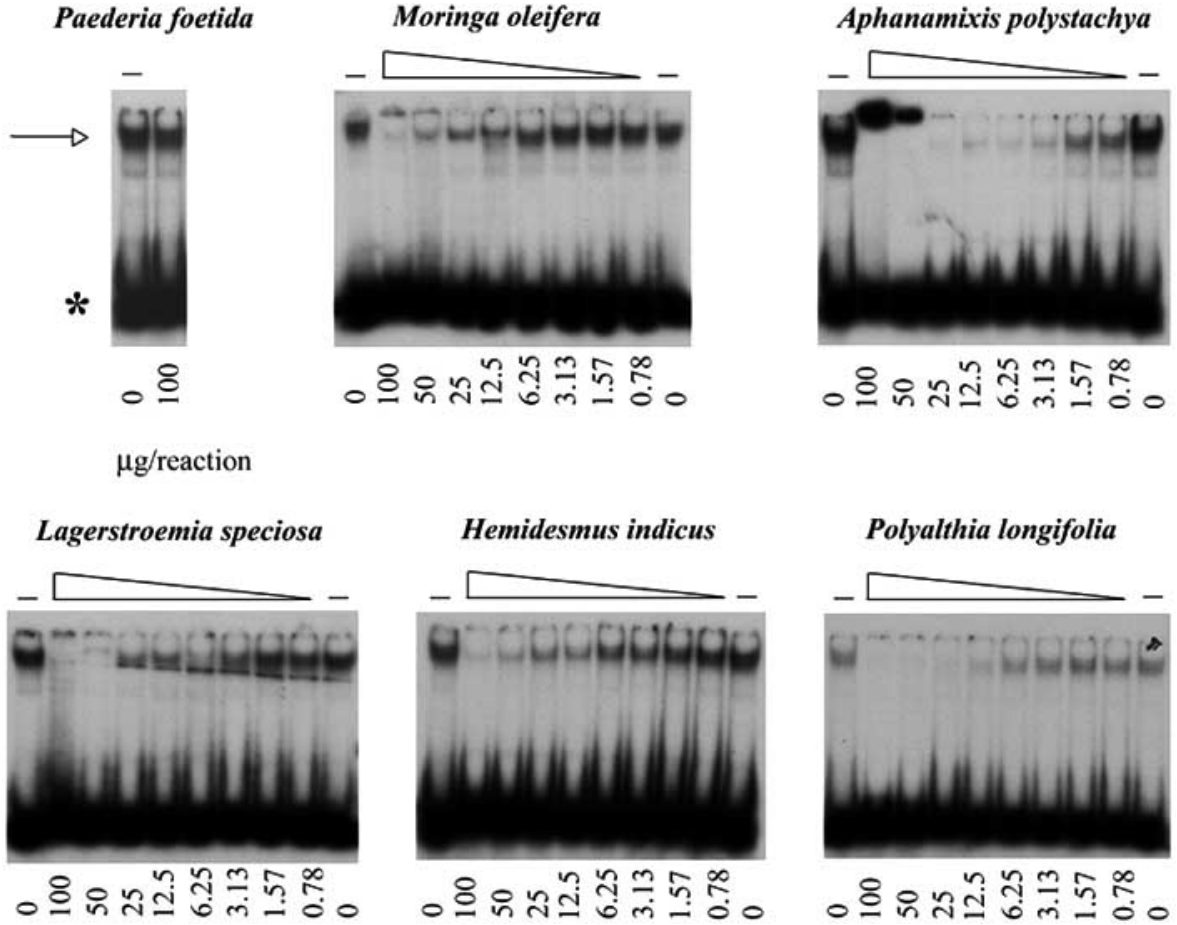

Fig. (3). Effects of different amounts (μg/reaction) of extracts from *Paederia foetida, Moringa oleifera, Aphanamixis polystachya, Lagerstroemia speciosa, Hemidesmus indicus* and *Polyalthia longifolia* on NF-kB/DNA interactions. * = free probe; arrows indicate NF-kB/DNA complexes.

Table 3. Effects of Extracts of Medicinal Plants in Inhibiting NF-kB TF/DNA Interactions

Medicinal plant	Inhibitory effect (IC_{50}, μg/ reaction)
Hemidesmus indicus	12.5 ± 4.5
Polyalthia longifolia	12.5 ± 2.8
Aphanamixis polystachya	0.78 ± 0.13
Moringa oleifera	12.5 ± 2.5
Lagerstroemia speciosa	25.0 ± 7.8
Paederia foetida	>100
Cassia sophera	>100
Hygrophila auricolata	>100
Ocinum sanctum	>100

Data represent the average ± SD from densitometric analysis obtained on autoradiographies exposed for three different lenght of time.

inhibit also NF-kB/DNA interaction. Accordingly, some extracts exhibiting low antiproliferative activity do not inhibit NF-κB/DNA interaction (for instance *Hemidesmus indicus* and *Polyalthia longifolia*). We like however to point out that some extracts do not inhibit NF-κB/DNA interactions, despite being highly effective in inhibiting cell proliferation. These data support the hypothesis that the molecular mechanism leading to antiproliferative activity of the analyzed plant extracts are several and different bioactive compounds present within the extract are probably present.

DISCUSSION

Natural products have been in the recent past considered as a source of bioactive molecules, facilitating drug discovery programs.

In this respect, characterization of biological activities of extracts from medicinal plants is very important for research projects aimed at the identification of bioactive molecules.

As elsewhere reviewed, targeting the transcription factor machinery is one of the most interesting approaches leading to control of gene expression [38]. This is firmly demonstrated by the effects of DNA-binding compounds for the development of anti-tumor and anti-viral molecules [39, 40]. A second set of experimental data on the clinical relevance of transcription modifiers is related to the use of decoy molecules targeting transcription factors [41-44].

As far as application of medicinal plant extracts for the control of gene expression, several reports are available in the literature [45-48]. For instance, Amri *et al*., using pharmacological, biochemical and proteomic methods, demonstrated that the standardized Ginkgo biloba extract EGb 761 and its isolated component ginkgolide B (GKB) inhibit peripheral-type benzodiazepine receptor (PBR) ligand binding and protein expression. This was due to a decrease of PBR mRNA-levels, due to transcriptional suppression of PBR gene expression. Further studies indicated that the action of GKB is mediated by a transcription factor binding to the PBR gene promoter, thereby regulating PBR gene expression [48].

As expected, several mechanism of action of isolated compounds from medicinal plants have been reported, such as direct interactions and modification of target transcription factors [49,50].

We have in this study focused on the effects of extracts from Bangladeshi medicinal plants on interactions between NF-κB transcription factors and target DNA sequences.

Among the plant extracts analyzed, those obtained from *Hemidesmus indicus, Polyalthia longifolia, Moringa oleifera, Lagerstroemia speciosa* and *Aphanamixis polystachya* inhibit NF-kappaB/DNA interactions and should be further considered for identification of bioactive compounds.

This research is in our opinion of interest, since NF-kappaB is involved in several pathologies, including rheumatoid arthritis [36], chronic asthma [37] and inflammatory bowel diseases (IBD) [31-33]. In addition, NF-κB is involved in the pro-inflammatory stage of cystic fibrosis, as reported in several studies [51-54]. NF-κB mediated induction of pro-inflammatory genes leads to the synthesis of cytokines, adhesion molecules, chemokines, growth factors and enzymes. Therefore the complex NF-κB pathway offers a variety of potential molecular targets for chemotherapeutic intervention [55-57].

In this respect, extracts from *Hemidesmus indicus, Polyalthia longifolia, Moringa oleifera, Lagerstroemia speciosa* and *Aphanamixis polystachya* could be a potential source in future experiments aimed at the identification of molecules of possible therapeutic relevance in the treatment of these NF-kappaB related diseases. In this study, indeed, the plant extracts have not been characterized with respect to their composition (such as lipids, peptides and low-molecular weight compounds).

As far as applications, we would like to point out that molecules inhibiting NF-κB binding activity are expected to be used for the control of proliferation of tumor cells [29,58,59]. In this respect the antiproliferative activity of *A.polystachia*, *L.speciosa* and *M.oleifera* might be caused, at least in part, by inhibition of NF-κB/DNA interactions.

On the other hand, *H.indicus* and *P.longifolia* extracts inhibit NF-κB/DNA interactions with much lower effects on cell growth. These extracts could be of interest in the search of compounds active in inflammatory diseases, for which inhibition of NF-κB activity without toxic effects should be obtained.

In conclusion, the data presented in this report sustain the concept that extracts from medicinal plants can be employed to identify agents inhibiting the molecular interactions between transcription factors and DNA.

EXPERIMENTAL

Materials and Methods

The synthetic oligonucleotides utilized in this study were purchased from Sigma Genosys (Sigma Genosys, Cambs, UK)

Plant Materials and Extraction

The stem bark of *Cassia sophera* was extracted with absolute ethanol in cold extraction process and the yield was 10.52%. The seeds of *Hygrophilla auriculata* were extracted with 80% ethanol:water in cold extraction process and the yield was 1.15%. The dried leaves of *Ocimum sanctum* were extracted with absolute ethanol in cold extraction process and the yield was 3.22%. The dried whole plant of *Lagerstroemia speciosa* was first defatted with petroleum ether and then extracted with chloroform and the yield was 7.1%. The roots of *Hemidesmus indicus* Linn, the stem barks of *Polyalthia longifolia* (Sonn.) and the stem barks of *Aphanamixis polystachya* Wall & Parker were extracted with 80% ethanol:water in cold extraction process. The yield of the first one was 8.9%, the second was 6.87% and the yield of the third, which resulted in two extracts, an oily phase and a solid mass, was 2.54% and 8.91%, respectively. The whole plant *Paederia foetida* Linn. (yield 11.92%) and the roots of *Moringa oleifera* Lamk. were extracted with absolute ethanol in a cold extraction process. The crude extract of *Moringa oleifera* (yield 14.54%) was further partitioned with *n*-hexane (yield 7.39%), chloroform (yield 5.94%), methanol (yield 9.46%) and water (yield 9.48%).

Cell Lines, Culture Conditions and Assays of *In Vitro* Antiproliferative Activity

Human erythroleukemia K562 cells [27] were cultured in a humidified atmosphere at 5% CO_2, in RPMI 1640 (Flow laboratories, Irvine, UK) supplemented with 10% fetal bovine serum (FBS; CELBIO, Milano, Italy), 100 units/ml penicillin and 100 μg/ml streptomycin (Aldrich, St.Louis, MA, USA). Cell number/ml was determined by using a model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL, USA). Usually, cells were seeded at the initial cell concentration of $3x10^4$ cells/ml and the cell number/ml determined after 2, 3, 4, 5 days of cell culture. IC_{50} was determined usually after 4 days, when untreated cells are in the log phase of cell growth [27].

Electrophoretic Mobility Shift Assay (EMSA)

Electrophoretic mobility shift assay (EMSA) was performed by using double stranded ^{32}P-labelled oligonucleotides as target DNA. Binding reactions were set up as described elsewhere in binding buffer (10% glycerol, 0.05% NP-40, 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5 mM DTT, 10 mM $MgCl_2$), in the presence of poly(dI:dC)·poly(dI:dC) (Pharmacia, Uppsala, Sweden), 2-5 μg of crude nuclear extracts and 0.25 ng of labelled oligonucleotide, in a total volume of 20 μl [41,42]. After 30 min binding at room temperature samples were electrophoresed at constant voltage (200 V for 1 hr) through a low ionic strength (0.25 x TBE buffer) (1 x TBE = 0.089 M Tris-borate, 0.002 M EDTA) on 6% polyacrylamide gels until tracking dye (bromophenol blue) reached the end of a 16 cm slab. Gels were dried and exposed for autoradiography with intensifying screens at -80°C. In these experiments, DNA/protein complexes migrate through the gel with slower efficiency. In studies on the inhibitors of protein/DNA interactions, addition of the reagents was as follows: (a) poly(dI:dC)·poly(dI:dC); (b) labelled oligonucleotides mimicking the binding sites for transcription factors to be modulated; (c) plant extracts; (d) binding buffer; (e) nuclear factors. The nucleodide sequence of double-stranded target DNA utilized in these experiments was 5'-CGC FGG GGA CTT TCC ACG G-3' (sense strand, NF-κB) [41]. Specificity of interactions with NF-κB was confirmed by competition experiments using double-stranded oligonucleotides recognizing other transcription factors, including Sp1, MEF-2, GATA-1, STAT-3, CREB, AP-1 (data not shown).

In order to determine IC values, the autoradiograms exposed for 4, 12 and 24 hours were scanned using the BIO-RAD Gel Doc 2000 and the proportion of NF-kB binding activity of EMSA reactions performed in the presence of plant extracts was compared to that of untreated controls.

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ABBREVIATIONS

NF-κB = Nuclear Factor-kappaB

TF = Transcription Factor

EMSA = Electrophoretic Mobility Shift Assay

IC = Inhibitory Concentration

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