The Evaluation of Nitric Oxide Scavenging Activity of Certain Herbal Formulations in vitro: A Preliminary Study

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INTRODUCTION

The role of nitric oxide (NO) in numerous disease states has generated a considerable discussion over the past several years since the journal Science named it the molecule of the year in 1992. NO is an important bio-regulatory molecule, which has a number of physiological effects including control of blood pressure, neural signal transduction, platelet function, antimicrobial and antitumor activity. Low concentrations of NO are sufficient, in most cases, to effect these beneficial functions. However, during infections and inflammations, formation of NO is elevated and may bring about some undesired deleterious effects (Marcocci et al., 1994a,b).

The NO does not interact with the bioorganic macromolecules such as the DNA or proteins directly. However, in the aerobic conditions, the NO molecule is very unstable and reacts with the oxygen to produce, intermediates such as NO₂, N₂O₃, NO₃ the stable products nitrate and nitrite (Marcocci et al., 1994a,b) and peroxynitrite when reacted with superoxide (Levi, 1987; Wink et al., 1991). These products progenitors are highly genotoxic, the deamination of guanine, cytosine and adenine is mediated primarily by the N₂O₃. In addition to the formation of nitrosoamines and deamination of the DNA bases, recent studies indicate that the NO may also act by affecting the enzymatic activities of several thiol rich DNA repair proteins like DNA alkyl transferase, formamopyrimidine-DNA glycosalase and the DNA ligase that play a critical role in the maintenance of the genetic integrity (Wink et al., 1991).

The formation of carcinogenic N-nitroso compounds, deamination, oxidation of the DNA bases and inhibition of the critical DNA repair protein leads to mutagenesis and an initiation towards the process of carcinogenesis (Liu and Hotchkiss, 1995). There is now increasing evidence to suggest that NO and its derivatives produced by the activated phagocytes may have a genotoxic effect and may contribute in the multistage carcinogenesis process (Wink et al., 1991). Direct link between chronic inflammation and induction of cholangiocarcinoma has been found in the infections by the parasites such as Opisthorchis viverrini (liver fluke) (Haswell-Elkins et al., 1994). The continuous exposure to free radicals generated from the chronic inflammation has been found to cause more cancers than environmental chemicals (Ames and Gold, 1990).

Herbal drugs have been used by mankind since time immemorial to treat various disorders and offer an alternative to the synthetic compounds, as they have been considered either non-toxic or less toxic. The traditional Indian system of medicine, Ayurveda is based on the principle of balance and counter balance. Ayurveda (Ayu = life, Veda = knowledge) extensively uses the plant-derived compound formulations for the treatment of various ailments after a careful study into the type of the disease. Plants are complex mixtures of compounds and no single compound can provide the desired activity. Some compounds potentiate a desired therapeutic action, while others reinforce the same and yet others interact to neutralize and counteract any possible side effects that may exist. Therefore, several plants with the common desired activities and varied undesirable activities are selected so that the final formulation will have a concentrated desired activity

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and the undesired activities will be diluted or absent altogether. The mechanism of action of herbal drugs and their extract preparations differ in many respects from that of the synthetic drugs or single substances (Wagner, 1999). It can be characterized as a polyvalent action and interpreted as additive or, in some cases, potentiating. Further, it has also been observed that in such formulations, certain other compounds may be of help in enhancing the potency of the active compounds resulting in an additive or synergistic positive effect, which in its total final gives immense benefit to the patient (Kulkarni, 1997). Since excess generation of NO is deleterious to human health, it is desired to screen certain herbal formulation that are commonly used in India for their capability to inhibit nitric oxide generation in vitro.

**MATERIALS AND METHODS**

The NO scavenging activity was determined in vitro using abana, geriforte, septilin, triphala, chyavanaprasha and mentat.

**Composition of abana.** The abana is a mixture of the following medicinal plants in definite proportions Asparagus racemosus, Terminalia arjuna, Withania somnifera, Tinospora cordifolia, Centella asiatica, Terminalia chebula, Glycyrrhiza glabra, Phyllanthus emblica, Boerhaavia diffusa, Convolvulus pluricaulis, Ocimum sanctum, Nardostachys jatamansi, Piper longum, Carum copticum, Zingeribe officinale, Cyperus rotundus, Acorus calamus, Nepata hindostiana, Embelia ribes, Syzygium aromaticum, Celastrus paniculatus, Santalum album, Elellaria cardamomum, Aloe vera, Daucus carota, Foeniculum vulgare, Rosa damascena, Cinnamomum cassia, Crocus sativus, Nelumbium speciosum, Punica granatum, Pyrus malus and Eclipta alba.

**Composition of geriforte.** Geriforte contains Achillea millefolium, Adhatoda vasica, Allium cepa, Allium sativum, Pium graveolens, Argyria speciosa, Asparagus adscendens, Asparagus racemosus, Berberis aristata, Boerhavua diffusa, Caesalpinia digyna, Capparis spinosa, Carum copticum, Cassia occidentalis, Celastrus paniculatus, Centella asiatica, Cichorium intybus, Cicer arietinum, Coriandrum sativum, Crocus sativus, Curcuma longa, Cyamopsis psoralioides, Daucus carota, Eclipta alba, Elettaria cardamomum, Emblica officinalis, Embelia ribes, Foeniculum vulgare, Glycyrrhiza glabra, Mucuna pruriens, Myristica fragrans, Phyllanthus amarus Piper longum, Psidium guayava, Raphanus sativus, Solanum nigrum, Sphaeranthus indicus, Syzygium aromaticum, Tamarix gallica, Terminalia arjuna, Terminalia chebula, Tinospora cordifolia, Tribulus terrestris, Vitis vinifera and Withania somnifera, in definite proportions.

**Composition of septilin.** Septilin is a mixture of plants like Balsamodendron mukul Hook. Ex Stocks, Tinospora cordifolia (Wild.) Miers, Rubia cordifolia Linn., Embelica officinalis Gaertn., Moringa pterygosperma Gaertn. (also known as Moringa oleifera Lam. Syn. M.) and Glycyrrhiza glabra Linn. in definite proportions.

**Composition of triphala.** The formulation, triphala is a mixture of Terminalia chebula, Terminalia bellerrica and Phyllanthus emblica Linn or Emblica officinalis in equal proportions (1:1:1).

**Composition of chyavanaprasha.** The drug chyavanaprasha is a mixture of Emblica officinalis, Agele marmelos, Clerodendrum phlomoidis, Oroxylum indicum, Gmelina arborea, Stereospermum suaveolens, Sida cordifolia, Desmodium gangeticum, Uaria picta, Teramnus labialis, Piper longum, Tribulus terrestris, Solanum indicum, Solanum xanthocarpum, Pistacia integerrima, Phaseolus trilobus, Phyllanthus niruri, Vitis vinifera, Leptadenia reticulata, Inula racemosa, Aquilaria agallocha, Tinospora cordifolia, Terminalia chebula, Elettaria cardamomum, Habenaria intermedia, Microstylis wallichii, Microstylis museifera, Hedychium spicatum, Cyperus rotundus, Boerhavia diffusa, Polygonatum verticillatum, Nympheae alba, Santalum album, Pueraria tuberosa, Adhatoda vasica, Roscoea alpina, Martynia diandra and Sesamum indicum in definite proportions.

**Composition of mentat.** The drug mentat is a mixture of Bacopa monnieri, Centella asiatica, Evolvulus alsinoides, Valeriana wallchii, Prunus amygdalus, Acorus calamus, Oroxylum indicum, Mucuna pruriens, Elettaria cardamomum, Foeniculum vulgare, Ipomea digitata, Orchis mascula, Zingeribe officinale, Celastrus paniculatus, Tinospora cordifolia, Emblica officinalis, Terminalia arjuna, Withania somnifera, Nardostachys jatamansi, Embelia ribes, Terminalia bellerica, Terminalia chebula, Myristica fragrans, Syzygium aromaticum in definite proportions.

**Preparation of the extract.** 100 g of the commercially available abana, geriforte, mentat, septilin, chyavanaprasha and triphala (Himalaya Drug Co., Bangalore, Dabur India Ltd., New Delhi, and Zandu Pharmaceuticals, Bombay, India, respectively), was extracted in 50% ethanol (1 l) at 50 to 60 °C in a Soxhlet apparatus for 120 h. The cooled hydroalcoholic liquid extracts were concentrated by evaporating its liquid contents. The aqueous extract of triphala powder was prepared (Jagetia et al., 2002). Briefly, 100 g of the triphala powder (Zandu Pharmaceuticals, Bombay, India) was boiled in 1000 ml of DDW till the volume was reduced to one fourth of the original (250 ml). The extract was cooled, centrifuged (Sorvall RC-5B, USA), the supernatant was collected and concentrated by evaporating its liquid contents.

**Nitric oxide generation and assay of nitric oxide scavenging.** Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction as described previously. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide (Green et al., 1982; Marconi et al., 1994a,b), which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (Marconi et al., 1994a,b). Sodium nitroprusside (5 mM) in phosphate-buffered saline was mixed with different concentrations of the drugs dissolved in the suitable solvent systems and incubated at 25 °C for 150 min. The samples from the

Table 1. Nitric oxide scavenging of certain herbal formulations

<table>
<thead>
<tr>
<th>Drug Conc. (µg/ml)</th>
<th>Gingko biloba (95% Et.)</th>
<th>Abana (50% Et.)</th>
<th>Chyavanprash (50% Et.)</th>
<th>Geriforte (50% Et.)</th>
<th>Mentat (50% Et.)</th>
<th>Septilin (50% Et.)</th>
<th>Triphala (Aq.)</th>
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<tr>
<td>0</td>
<td>—</td>
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<tr>
<td>31.25</td>
<td>22.08 ± 4.02</td>
<td>70.83 ± 0.58</td>
<td>52.53 ± 5.93</td>
<td>59.30 ± 3.96</td>
<td>48.71 ± 1.54</td>
<td>63.59 ± 1.20</td>
<td>53.77 ± 1.19</td>
</tr>
<tr>
<td>62.5</td>
<td>36.22 ± 2.77</td>
<td>72.54 ± 1.66</td>
<td>55.38 ± 2.98</td>
<td>69.19 ± 1.58</td>
<td>59.13 ± 1.36</td>
<td>68.29 ± 0.64</td>
<td>61.91 ± 2.56</td>
</tr>
<tr>
<td>125</td>
<td>48.45 ± 3.48</td>
<td>74.52 ± 1.46</td>
<td>65.27 ± 2.25</td>
<td>69.93 ± 1.03</td>
<td>61.45 ± 1.21</td>
<td>69.66 ± 0.13</td>
<td>70.03 ± 1.26</td>
</tr>
<tr>
<td>250</td>
<td>54.92 ± 2.13</td>
<td>79.98 ± 1.87</td>
<td>68.45 ± 2.35</td>
<td>66.61 ± 0.73</td>
<td>67.21 ± 2.21</td>
<td>68.82 ± 1.66</td>
<td>70.16 ± 0.89</td>
</tr>
<tr>
<td>500</td>
<td>55.57 ± 1.81</td>
<td>80.72 ± 1.65</td>
<td>74.66 ± 1.83</td>
<td>52.50 ± 3.31</td>
<td>55.04 ± 0.32</td>
<td>54.98 ± 2.40</td>
<td>62.28 ± 1.09</td>
</tr>
<tr>
<td>1000</td>
<td>45.36 ± 2.20</td>
<td>80.49 ± 3.23</td>
<td>74.39 ± 1.95</td>
<td>60.54 ± 0.55</td>
<td>62.69 ± 1.31</td>
<td>60.07 ± 3.87</td>
<td>67.78 ± 2.01</td>
</tr>
</tbody>
</table>

Aq, aqueous; Et, ethanolic; — denotes no scavenging.

above was reacted with Griess reagent (1% sulphanilamide, 2% H₃PO₄ and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was read at 546 nm and referred to the absorbance of standard solutions of potassium nitrite treated in the same way with Griess reagent.

RESULTS

The results are shown as percent NO scavenging in Table 1.

All the polyherbal extracts exhibited a good NO scavenging activity in vitro and the scavenging activity was better than *Gingko biloba*, the positive control used. The 50% ethanolic extract of abana showed a concentration dependent NO scavenging that reached a peak of 80.72% at 500 µg/ml and remained unaltered thereafter. The scavenging activity of 31.25 µg/ml of abana was 3.21 fold greater than that of the concurrent concentration of *G. biloba* (Table 1).

The chyavanaprasha extract also showed a dose dependent elevation in NO scavenging activity up to 1000 µg/ml (74.66%) and the NO scavenging was 1.64 fold greater than that for *G. biloba* at the equimolar concentration, (55.57%) (Table 1).

The ethanolic extract of geriforte showed a concentration dependent elevation in NO scavenging activity and the best activity was seen at a low dose of 125 µg/ml where 69.93% scavenging was observed. With the further increase in the drug concentration, a gradual decline in NO scavenging activity was observed. The NO scavenging of geriforte was 1.44 fold greater than that of *G. biloba* at an equimolar concentration of 125 µg/ml, where 48.45% scavenging was observed for *G. biloba*, as against the 69.93% for geriforte extract (Table 1).

The extract of mentat showed a concentration dependent elevation in NO scavenging activity up to 250 µg/ml (67.21%), which was 1.22 fold higher than the equimolar concentrations of *G. biloba* (54.92%). However, a decline was observed thereafter at the higher doses studied (Table 1).

The extract of septilin inhibited the generation of NO in a concentration dependent manner up to 125 µg/ml (69.66%) and a gradual decline thereafter at the higher doses. The NO scavenging was 1.43 fold greater than the respective dose of *G. biloba*, where 48.45% scavenging was observed at 125 µg/ml (Table 1). The aqueous extract of triphala also caused a concentration dependent elevation in the scavenging of NO in vitro. A peak scavenging of 70.16% was observed at a low dose of 125 µg/ml, which was 1.6 fold higher than that of *G. biloba* at the equimolar concentration. The scavenging activity of triphala remained unaltered up to 250 µg/ml and showed a decline thereafter (Table 1).

DISCUSSION

Nitric oxide is an essential bioregulatory molecule required for several physiological processes like neural signal transmission, immune response, control vasodilation and control of blood pressure, (Palmer et al., 1987; Rees et al., 1989; Bredt and Snyder, 1990; Gold et al., 1990) etc. However, the elevation of the NO results in several pathological conditions, including cancer. NO is a short-lived (half-life 3–30 s) colorless gas that is moderately soluble in water (up to 2 mmol/L) but highly soluble in organic solvents (Ignarro et al., 1987; Nathan, 1992). It is lipophilic in nature and can diffuse between cells very easily. NO is generated from the terminal guanido nitrogen atom of L-arginine by various NADPH-dependent enzymes called NO synthases (NOS), (Moncada et al., 1991). The three main isoforms are neuronal (n) NOS, inducible (Lala, 1998) NOS, and endothelial (e) NOS. Generally, nNOS and eNOS are expressed constitutively in neurons and endothelial cells, respectively, though they can also be expressed by other cells. NO has an unpaired electron, hence is a free radical (NO). NO becomes nitrosonium cation (NO⁺) or nitroxyl anion (NO⁻) by donating or accepting an electron, respectively (Nathan and Xie, 1994). NOS is synthesized in a variety of cell types from multiple mammalian species and can produce consistent, high concentrations of NO upon induction with cytokines and or bacterial lipopolysaccharide (LPS) (Nathan and Xie, 1994).

The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in vivo. The extracts of polyherbal formulation like chyavanaprasha, abana, triphala, septilin, geriforte and mentat reduced the generation of NO in vitro in a concentration dependent manner. This reduction was 1.64 and 1.77 fold greater for chyavanaprasha and abana, when compared to the
equimolar concentration of 1000 µg of *Ginkgo biloba*, which was used as positive control. Similarly, the extracts of triphala and mentat inhibited the NO generation in vitro and this activity was 2.25 and 2.43 folds greater than the equimolar concentration of *Ginkgo biloba* at 31.25 µg. The extracts of septilin and geriforte reduced NO generation by 2.88 and 2.68 folds at 31.25 µg, when compared with the concurrent *Ginkgo biloba* control. The reports regarding the inhibition of NO generation by these polyherbal preparations are unavailable. However, the crude extracts of certain plants like *Ginkgo biloba* (Marcocci et al., 1994a,b), *Sanguisorbae Radix*, *Caryophylli Flora*, *Copсидis Rhizoma*, *Granati Cortex*, *Gallae Rhizis*, *Rhei Rhizoma* and *Cinnamomii Cortex* have been reported to inhibit NO generation in vitro (Yokozawa et al., 2000). Similarly, other natural agents like vitamin C (Fraga et al., 1991), β-carotene (Arroyo et al., 1992), epigallocatechin, carnosol (Chan et al., 1995), different curcuminoids (Chan et al., 1995; Sreejayan and Rao, 1997), pycnogenol (Virgili et al., 1998), coumarins (Miyake et al., 1999), phenyletanoids (Xiong et al., 2000) and sanguinin H-6 (Yokozawa et al., 2002) have been reported to inhibit or mitigate the adverse effects of NO. The implications of these findings may be very important for human health, since these drugs have been used in India from ancient times. Further, the high scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO, that are detrimental to the human health.

Some of the polyherbal formulations used in this study have been used since time immemorial to treat various disorders. Chyawanprashra and triphala are arguably the most commonly used rasayana drugs (rejuvenators) in Ayurveda, and have been reported to scavenge free radicals (Vaidya et al., 1998). Their additional property to scavenge NO may add to its effectiveness as a rejuvenator. Similarly geriforte and septilin are reported to be immunomodulators and adaptogenic and their efficacy in the NO scavenging may partly be responsible for their clinical activity, as excess NO is known to damage the immune system and deteriorate health.

Mentat a neurotonic and abana a cardiotonic were also observed to scavenge NO and may have great implications in the *in vivo* systems in reducing the NO induced damage to the macromolecules. Detailed *in vivo* studies are planned to explore the efficacy of these drugs in ameliorating the NO induced damage.

**Acknowledgements**

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**REFERENCES**


