CHAPTER IV

*IN VITRO* ANTI-OXIDANT ACTIVITY OF SYNTHESISED HETEROCYCLICS

A. ANTIOXIDANT ACTIVITY EVIDENCED TO POSSESS ANTICANCER ACTIVITY

Oxidative stress is one of the major causes of increased risk of cancer and cardiovascular disease. The prevention and treatment of these diseases through the use of exogenous antioxidants from synthetic origin has received increasing interest in recent years.¹

The efficiency of antioxidants in preventing cancer should be differentiated from the contribution of antioxidants during anticancer therapy. For example, Lycopene can inhibit the development of prostate cancer but it reduces the ability of radiation to destroy prostate cancer cells.

On the other hand another anti-oxidant Vitamin-E seems to promote the destruction of cancer cells by reduction *in vitro* and to enhance the normal healing of tissue following completion of radiation therapy.

Paradoxically treatment with high doses of Vitamin-C can inhibit cancer cell division and increase the sensitivity of tumours to radiotherapy. The effects of antioxidants are multi-factorial and highly dependent on initial conditions. Their actions are indeed a tangled web.

Synthetic and naturally occurring antioxidants have a wide variety of biological actions in addition to their primary antioxidant activity. Some of the included biological effects are of direct interest in relation to studies of carcinogenicity and / or modulation of carcinogenesis. Since the synthetic
antioxidant BHA was first found to exert carcinogenic potential in rat and hamster forestomach epithelium, many other synthetic and naturally occurring antioxidants have been examined for their ability to induce proliferative activity in the alimentary canal.

The use of antioxidants along with chemotherapy in prostate, breast, lung and colon cancer patients is scientifically evidenced to improve drug effectiveness or may reduce side effect severity.

Therefore chemotherapy drugs that cause high levels of “oxidative stress" are thought to rely in part on oxidative stress to kill cancer cells. This is because oxidative stress slows cell replication. However chemotherapy relies on fast cancer cell replication to be effective because it is during the moment of cell replication that chemotherapy kills cancer cells.

One approach to addressing this problem is the addition of certain antioxidants at specific dosages to lessen oxidative stress, thus making chemotherapy treatment more effective.²⁻⁶

MECHANISMS BY WHICH ANTI-OXIDANTS PLAY A ROLE IN CANCER THERAPY

1. Activation of AMPK (AMP- activated Protein Kinase) - [ By exercise and fasting - inhibit growth of some cancer (including colon cancer)].
2. Activation of Bax [lead to cell death]- e.g.: genistein from soya .
3. Activation of caspase-3 [ promotes cell death].
4. Inhibition of cyclooxygenase-2 (Cox-2) [inhibited by using long term NSAIDS, decreases the risk of colorectal cancer], eg: Curcumin.
5. Regulation of C-Myc Protein
6. Decreases Glut-1, a transporter Protein which transports glucose into tissues, eg: Green tea

7. Activation of P53 & P21 in colon cancer [suppresses tumour growth]. eg: Vitamin-E.⁷

Moreover the anticancer effect of honey may be attributed to its antioxidant activity. An enhanced antioxidant status with apoptosis has been observed in hepatocellular carcinoma cells. Daily consumption of 1.2 g / kg.b.w of honey has been shown to elevate the amount and the activity of antioxidant agents such as beta-carotene, vitamin-C, glutathione reductase and uric acid.⁸

More research is needed to improve our understandings of the positive effect of antioxidants and cancer. It is necessary to assess the anti-oxidant property by standard methods before defending the anti-cancer activity in specific cancer cell lines.

B. LITERATURE ENVISAGED FOR ANTI-OXIDANT ACTIVITY OF SELECTED HETEROCYCLICS

1. Hocman. G et al., (1988)⁹ revealed that synthetic phenolic antioxidants (e.g. BHT, BHA) are preventing cancer either via interception of harmful free radicals, activating the detoxifying enzymes of the body, inhibiting the formation of ultimately carcinogenic metabolites and their binding to DNA, and modifying the immune response of the organism.

2. Shi. X et al., (2000)¹⁰ revealed that Pyrrolidine dithiocarbamate (PDTC) is considered an antioxidant and is frequently used to study the role of free radical reactions in various biological processes and against free radical-induced cellular injuries. In this study, electron spin resonance (ESR) was
used to investigate the antioxidant potential of PDTC with hydroxyl radical (*OH) and superoxide anion radicals (O2*-).

3. Adeyemo. D et al., (2001)\textsuperscript{11} revealed that 5 Fluorouracil (5-FU), the most effective systemic chemotherapeutic agent in the management of advanced colorectal carcinoma acts by inducing apoptosis. This suggests that reduction of intracellular levels of reactive oxygen species enhance susceptibility to 5-FU (apoptotic stimuli) by augmentation of bax expression.

4. Simon Ching et al.,(2002)\textsuperscript{12} analysed blood samples from 150 women newly diagnosed with breast cancer and 150 women with no history of breast cancer. High amounts of total antioxidants in the blood stream were much more common in women without breast cancer. High concentrations of beta-carotene appeared particularly protective.

5. Shimakawa et al., (2003)\textsuperscript{13} explored the anti-oxidant of lipophilic nitroxy radical, cyclohexane - 1 - spiro - 2’ (4’ – oxyimidazolidine - 1’- oxyl) -5’- spiro - 1’’-cyclohexane against peroxidation under hypoxic conditions and reported that spiro compounds have been recently used as anti-oxidants.

6. Drisko JA et al., (2003)\textsuperscript{14} concluded that antioxidants and natural compounds can improve the effectiveness of radiotherapy and chemotherapy, with reference to over 4000 references to scientific papers and basically concludes that, far from in some way interfering with the biochemical process involved in chemo or radiotherapy, taking anti-oxidants actually improves the success rates of both.

7. Guddadarangavvanahally K et al., (2004)\textsuperscript{15} revealed the antioxidant activities of flavidin in different in vitro model systems viz., β-carotene-
Linoleate, 1,1-diphenyl-2-picryl hydrazyl (DPPH), Phospho molybdenum method and scavenging of hydrogen peroxide methods. The data obtained in the \textit{in vitro} models clearly established the antioxidant potency of flavidin.

8. Chanda. S \textit{et al.}, (2009)\textsuperscript{16} reviewed \textit{in vitro} models like total phenolic content, total flavonoid reducing power, \textit{free radical scavenging assays}, superoxide anion radical scavenging assay, xanthine oxidase and \textit{Nitric oxide Scavenging assay} etc for anti-oxidant activity evaluation and some medicinal plants possessing anti-oxidant properties.

9. Mohamed Youssef \textit{et al.}, (2010)\textsuperscript{17} reported microwave assisted of some new heterocyclic \textit{Spiro-derivatives} using homophthalic anhydride & aromatic amines with potential antimicrobial and antioxidant activity.

10. Sarma. B. K \textit{et al.}, (2010)\textsuperscript{18} reported the structure, \textit{spirocyclisation mechanism} and glutathione peroxidase like-anti oxidant activity of stable spirodiazaselenurane and spirodiazatellurane.

11. Wanyi Zhao \textit{et al.}, (2010)\textsuperscript{19} proposed a series of new bromophenols and chlorophenols which were prepared by a practical route. The \textit{in vitro} antioxidative activity of the halophenols was evaluated by the \textit{1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay}, and their cytoprotective activity was also tested on hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})-induced injury in human umbilical vein endothelial cells (HUVEC).

12. Omar A. Miqdad \textit{et al.}, (2011)\textsuperscript{20} reported the synthesis of antimicrobial activity of \textit{spirocompounds} via the reaction of hydrazonoyl halides with exocyclic 4-arylidene-2-methylimidazolin-5-one in benzene in the
presence of triethylamine and exhibited significant antibacterial activity compared with selected standard drugs.

13. Tarik E. Ali et al., (2014)\textsuperscript{21} reported a convenient synthetic approach leading to a series of novel substituted azoles, azines, and azepines linked to \textit{α-aminophosphonates} moiety was achieved. Also, evaluation of their antioxidant properties shows that the compounds having 1,5-benzoxazepinyl and 1,5-benzodiazepinyl units in combination with \textit{α-aminophosphonic diester} moiety are the most powerful antioxidant agents.

14. Ivan AL et al., (2014)\textsuperscript{22} proposed that Ultraviolet B (UVB) irradiation may cause oxidative stress- and inflammation-dependent skin cancer and premature aging. \textit{Pyrrolidine} dithiocarbamate (PDTC) is an antioxidant and inhibits nuclear factor-κB (NF-κB) activation.

Many FDA approved antioxidants potentiates the antitumor activity of chemo therapeutic drugs such as 5-FU and cyclophosphamide. It is known that oxidative stress is related to cancer. There is a strong correlation between the oxidative stress and the development of cancer. Many findings revealed that presumably because of the oxidative stress, carcinogenesis could be induced.\textsuperscript{23} To substantiate this, reports are available in developing potential candidates which has both antioxidant and anticancer property.

\textbf{C. AIM OF THE STUDY}

In this study, several spiro - oxindoles, pyrazole \textit{α- amino phosphonates} and acenaphthylene pyrrolidine heterocyclic scaffolds have been synthesised and screened for their antioxidant activity to support the earlier research and thus
creating a platform for treating oxidative stress may represent one of the novel approaches for the prevention and therapy of cancer.

In view of the considerable importance of the heterocyclics and its derivatives, the present work was aimed for testing of synthesised compounds for the anti-oxidant activity by using DPPH assay.

D. IN VITRO ANTI-OXIDANT ACTIVITY BY DPPH SCAVENGING ASSAY: (FREE RADICAL SCAVENGING ASSAY)

Free radicals have aroused significant interest among scientist in past decade, their broad range of effect in biological system have drawn on the attention of many experimental works. Free radicals are in the form of atoms or molecules or even may be an ion containing unpaired electrons which cause the oxidative tissue damage. Oxidative tissue damage is associated with cancer and other degenerative disorders. Hence antioxidants prevent the cell damage by scavenging these free radicals.

Principle

It is based on the measurement of the scavenging ability of synthesised compounds towards the stable DPPH. The odd electron in the DPPH gives a strong absorption maximum at 517 nm and is purple in colour. The colour turns from purple to yellow as the molar absorptivity reduces when the odd electron of DPPH becomes paired with a hydrogen from a antioxidant to form the reduced DPPH - H (2, 2 - diphenyl - 1 - picryl hydrazine). The resulting decolorization is stoichiometric with respect to number of electrons captured. More the decolorization more is the reducing ability.
DPPH• + H - A → DPPH – H + A+

\[
\begin{align*}
\text{N} & \quad \text{N} \\
\text{NO}_2 & \quad \text{NO}_2 \\
\text{NO}_2 & \quad \text{NO}_2 \\
\text{N} & \quad \text{N}
\end{align*}
\]

2, 2 - diphenyl - 1 - picryl hydrazyl
(radical form - purple)

2, 2 - diphenyl -1 - picryl hydrazine
(non- radical form - yellow)

Materials and Methods

i) Chemicals and solvents used

- 2, 2-diphenyl-1-picrylhydrazyl (DPPH) - [Sigma Chemicals, USA]
- Dimethyl sulphoxide (DMSO) - [Sigma Chemicals, USA]
- Ascorbic acid (Merck Ltd, Mumbai)
- Methanol
- Ethanol (Jiangsu Huaxi International Trade Co.Ltd, China).

ii) Instruments and accessories used

- 96 well plate : Nunc Nalgene, USA
- ELISA Reader : Bio rad, USA
- Incubator : Technico, India
- Weighing Balance: Sartorius, India.
iii) Preparation of the DPPH reagent

About 5.91 mg of DPPH was accurately weighed and dissolved in 100 ml of methanol (150 μM).

iv) Preparation of test and standard solution

Stock solution of 1000 μg/ml concentration of the synthesized test compounds (SV1 - 10, PV 1-10, VAZN1 -10) and standard ascorbic acid were prepared in 1% DMSO in ethanol. The stock solution was serially diluted with ethanol to obtain the required concentrations (1.97- 1000 μg/ml).

v) Procedure

The antioxidant activity of the test compounds and standard were assessed by the technique based on the DPPH scavenging ability using 96 well microtitre plate. To 190 μl of DPPH solution, 10 μl of each of the test sample and standard in the concentration range of 1.97- 1000 μg/ml was added separately in well of the microtitre plate. The plates were incubated at 37°C for 20 minutes and the decrease in absorbance of test compounds and standard (due to quenching of DPPH free radicals) were measured at 517 nm in ELISA Reader.

Absorbance of control blank, sample blank and control were measured as well. The experiment was performed in triplicates and the % scavenging activity was calculated using the below formula.
\[
\text{% Scavenging activity} = \frac{C - T}{C} \times 100
\]

Control (C) = C - CB, Test (T) = S - SB

where,

Control (C) = 10 µl methanol* + 190 µl DPPH

Control Blank (CB) = 10 µl diluents (1 % DMSO in ethanol) + 190 µl DPPH

Sample blank (SB) = 10 µl test solution + 190 µl diluents

Sample (S) = 10 µl sample solution + 190 µl DPPH

*Solvent used to dissolve DPPH.

The IC\textsubscript{50} values for each drug compounds as well as standard preparation were calculated. The effective concentration of sample required to scavenge DPPH radical by 50 % (IC\textsubscript{50} value) was obtained by linear regression analysis of dose – response curve plotting between % scavenging activity on y-axis and concentration on x-axis.
### E. RESULTS

#### Table 4.1. DPPH Free radical scavenging activity of spiro-oxindole analogues

<table>
<thead>
<tr>
<th>COMP ID</th>
<th>CONCENTRATION (µg/ml)</th>
<th>IC_{50} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.97</td>
<td>3.95</td>
</tr>
<tr>
<td>SV1</td>
<td>10.05±0.26</td>
<td>13.4±0.34</td>
</tr>
<tr>
<td>SV2</td>
<td>11.28±0.34</td>
<td>5.38±0.46</td>
</tr>
<tr>
<td>SV3</td>
<td>11.67±0.76</td>
<td>2.36±0.56</td>
</tr>
<tr>
<td>SV4</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>SV5</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>SV6</td>
<td>15.06±0.76</td>
<td>13.7±0.46</td>
</tr>
<tr>
<td>SV7</td>
<td>10.3±1.12</td>
<td>19.3±0.56</td>
</tr>
<tr>
<td>SV8</td>
<td>1.925±0.98</td>
<td>1.20±0.86</td>
</tr>
<tr>
<td>SV9</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>SV10</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>4.34±1.24</td>
<td>13.2±1.02</td>
</tr>
</tbody>
</table>
Fig : 4.1. DPPH free radical scavenging activities of spiro-oxindole analogues and the standard, ascorbic acid. The data represents the percentage inhibition on DPPH radicals with respect to concentration. All data expressed as mean ± SE (n=3).

Fig : 4.2. IC$_{50}$ values of Spiro-oxindole analogues on antioxidant activity by DPPH scavenging assay.
Table 4.2. DPPH Free radical scavenging activity of pyrazole α-amino phosphonates analogues

<table>
<thead>
<tr>
<th>COMP ID</th>
<th>CONCENTRATION (µg/ml)</th>
<th>IC_{50} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
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<tr>
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<td>PV10</td>
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</tr>
<tr>
<td>Ascorbic acid</td>
<td>4.34±1.24</td>
<td>13.28±1.02</td>
</tr>
</tbody>
</table>
Fig : 4.3. DPPH free radical scavenging activities of pyrazole \( \alpha \)-amino phosphonates analogues and the standard, Ascorbic acid. The data represents the percentage inhibition on DPPH radicals with respect to concentration. All data expressed as mean ± SE (n=3).

Fig: 4.4. IC\(_{50}\) values of pyrazole \( \alpha \)-amino phosphonates on anti-oxidant activity by DPPH scavenging assay.
Table 4.3. DPPH Free radical scavenging activity of acenaphthylene pyrrolidine analogues

<table>
<thead>
<tr>
<th>COMPOUND ID</th>
<th>CONCENTRATION (µg/ml)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1.97</td>
<td>3.95</td>
</tr>
<tr>
<td>VAZN1</td>
<td>2.4±1.08</td>
<td>3.2±1.46</td>
</tr>
<tr>
<td>VAZN2</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>VAZN3</td>
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<td>NI</td>
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<tr>
<td>VAZN4</td>
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<td>VAZN9</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>VAZN10</td>
<td>3.4±0.98</td>
<td>6.9±1.89</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>4.34±1.24</td>
<td>13.28±1.02</td>
</tr>
</tbody>
</table>
Fig : 4.5. DPPH free radical scavenging activities of acenaphthylene pyrrolidinone analogues and the standard, ascorbic acid. The data represents the percentage inhibition on DPPH radicals with respect to concentration. All data expressed as mean ± SE (n=3).

Fig: 4.6. IC$_{50}$ values of acenaphthylene pyrrolidinone on antioxidant activity by DPPH scavenging assay.
F. DISCUSSION

DPPH scavenging assay is considered as one of the most widely used *in vitro* methods to assess the antioxidant activity in a short span of time. The DPPH• accepts electrons or hydrogen radicals to form a stable diamagnetic molecule exhibiting the colour change from purple to yellow and the results are expressed as the ability of the DPPH• to undergo reduction at 540 nm.

a. The antioxidant potential of the *spiro-oxindole analogues* (SV1-SV10) were determined by DPPH method. The percentage antioxidant activity and IC₅₀ values of the synthesized compounds are shown in Table-4.1 and Fig - 4.1 & 4.2. Out of the ten compounds tested for their antioxidant activity, SV7 having allyl side chain showed potent free radical scavenging activity at 33.05 µg/ml, comparable with that of ascorbic acid at IC₅₀ 25.02 µg/ml. However, compound SV-8 having propagyl side chain at Nth position exhibited free radical scavenging activity with IC₅₀ at 93.36 µg/ml. Compounds of SV4 &SV9 bearing chloro substitution at the 5th position were found to be less potent when compared with the compounds SV7 & SV8 and their IC₅₀ value was found to be > 200 µg/ml. Thus, when the antioxidant activity of the parent system was conducted with their substitutions at the Nth position with allyl and propagyl groups in cyclopentano ring fused with oxindoles exhibited a greater increase in the antioxidant activity compared with cyclohexano ring substituents, as shown in the Table 4.1.

Further, it is inferred that the effect of other substituents such as, allyl (SV2), propagyl (SV3), chloro (SV4 & SV9) and ethyl acetate (SV5 & SV10) group at 5th and Nth position respectively were studied and a
paradoxical situation was observed; where, the above scaffolds unfortunately led to less anti oxidant activity as compared to its parent counterpart (SV1 & SV6).

b. The free radical scavenging activity of *pyrazole alpha amino phosphonates* analogues PV(1-10) was conducted and the effect of the different functional groups attached to amino (N-H) group such as p-chlorophenyl (PV1), o-chlorophenyl (PV2), o-bromophenyl (PV3), p-methoxyphenyl (PV4), o-methoxy p-nitrophenyl (PV6), m-methyl (PV7), benzyl (PV8), t-butyl (PV9), p-bromophenyl (PV10) in the phenyl parent scaffold (PV5) were studied. Among the ten compounds tested, PV4, PV7, PV8 have shown moderate scavenging activity and the rest of the seven analogues were deficient in activity by DPPH method. From the results (Table - 4.2, Fig - 4.3 & 4.4) it was inferred that PV4, the compound with p-methoxy phenyl substitution possessed moderate activity with 143.90 µg/ml, comparable with that of ascorbic acid at IC50 25.02 µg/ml. The compound bearing o-tolyl (PV7) and benzyl (PV8) substitutions shown temperate percentage inhibition of 43.4 ± 0.16 and 39.2 ± 0.06 respectively at the concentration of 1000 µg / ml.

The effect of substituents at para and meta positions of the phenyl ring was studied. Interestingly the radical scavenging ability of this scaffold was supported by the presence of moderately active methoxy group at the para position on the phenyl ring. However the presence of electron withdrawing group such as chloro,bromo at the ortho and para position did not favour the activity. But the compounds having chloro(PV2) and bromo (PV3) at ortho position responded to action when
compared to that of parent compound (PV5) and with that of compound having chloro (PV1) and bromo (PV10) at para position having no inhibition.

c. Ten compounds of **acenaphthylene pyrrolidine derivatives** (VAZN1-VAZN10) were tested for free radical scavenging activity. Their percentage inhibition activity and IC$_{50}$ values are shown in Table- 4.3, Fig - 4.5 & 4.6. Among the ten compounds tested, VAZN10 and VAZN1 have shown maximum scavenging activity with IC$_{50}$ 178.90 & 250 µg / ml respectively and the rest of the eight analogues were deficient in activity by DPPH method. From the result, it was inferred that the compound with dimethyl- methoxy phenyl (VAZN1) and diethyl- o,p - dimethyl phenyl (VAZN10) substitution possessed moderate activity comparable with that of ascorbic acid at IC$_{50}$ 25.02 µg / ml. The compound bearing dimethyl-o,p-dimethyl phenyl (VAZN5), dimethyl-tolyl (VAZN4) and diethyl-tolyl (VAZN6) substitutions shown temperate percentage inhibition of 48.26 ± 0.46, 40.70 ± 0.28 and 31.78 ± 0.98 respectively at the concentration of 1000 µg/ml.

The effect of substituents at ortho, para and meta positions of the phenyl ring with p - bromo (VAZN3 & VAZN7), m - methyl (VAZN4 & VAZN6), p - methoxy (VAZN1 & VAZN9) and o, p - dimethyl (VAZN5 & VAZN10) was studied with reference to the parent scaffold (VAZN2 & VAZN8) along with variation in the diacarboxylate substitutions with dimethyl (VAZN1 - 5) and diethyl groups (VAZN6 - 10). Interestingly the radical scavenging ability of this scaffold was supported by the presence of moderately active o, p dimethyl (VAZN10) and methoxy group at the para
position (VAZN1) on the phenyl ring. However the presence of electron withdrawing group such as bromo at para position (VAZN3 & VAZN7) did not favour the activity. But the compounds having m-tolyl substitution (VAZN4 & VAZN6) exhibited action when compared to that of parent compound (VAZN2 & VAZN8).

From the results of DPPH free radical scavenging activity of three different heterocyclics like spiro-oxindoles (SV1-10), pyrazole α-amino phosphonates (PV1-10), Acenaphthylene pyrrolidine (VAZN1-10), out of 30 compounds screened, only 13 compounds (SV7, SV8, SV5, SV1, SV6), (PV4, PV7, PV8) & (VAZN10, VAZN1,VAZN4,VAZN5,VAZN6) were found to be promising in inhibiting free radicals by DPPH method.

G. CONCLUSION

A functionally-validated *in silico* - *in vitro* approach to the reliable and efficient prediction of ligands binding to a cancer target is to be notable before proceeding to perform *in vitro* anti-cancer activity. Therefore at this juncture, there is a need for a potent combination of *in silico* (*Molecular docking study*) prediction to identify the promising ligands out of 30 synthesised compounds with realizing that prediction must also be verified *in vitro studies*. Therefore the study was to validate the utility of the approach by performing molecular docking studies and an additional scavenging mechanism using *Nitric oxide scavenging activity* (*anti-oxidant activity*) to accomplish that the prioritized ligands by both *in silico* and *in vitro* (DPPH) models are potent hunters in destroying cancer.
H. REFERENCES


5. Neuhouser ML, Patterson RE, Thornquist MD, Omenn GS, King IB, Goodman GE, Fruits and vegetables are associated with lower lung cancer risk only in the placebo arm of the b-Carotene and Retinol Efficacy Trial (CARET), *Cancer Epidemiol Biomarkers Prev*, 2003, 12, 350.


dithiocarbamate inhibits UVB-induced skin inflammation and oxidative stress in hairless mice and exhibits antioxidant activity *in vitro*.

