CHAPTER I
INTRODUCTION
CHAPTER 1

ISOLATION AND CHARACTERIZATION OF COMPOUNDS
FROM SEMECARPS ANACARDIUM LINN. NUT MILK EXTRACT

1.1 INTRODUCTION

Humans have gathered food and medical herbs ever since their arrival on earth. We were guided then by instinct, followed by experience and more recently, also by rational thought. For millions of years, mankind has fared quite well using this approach, but after the development of science and technology, many people felt that the current state of affairs was quite satisfactory and hence, they failed to support research and education adequately. Yet, the activities of humans on this clod evidently interact effectively with other evolving systems of nature, with consequences that may become very harmful to higher life soon. Therefore, it is time to examine more closely what we are eating, how diseases can be treated more rationally and how we can more effectively conserve our natural resources. Although the analyses of such problems at the moment are neither sufficiently diversified nor adequately penetrant, the feeling that such work is urgent has become widespread (Harborne, 1988a; Dixon et al., 1998; Montanari et al., 1998).

Plants are the basis of life on earth and are central to people’s livelihood (Sajem and Gosai, 2009). Plants have also provided man with all his needs in terms of shelter, clothing, food, flavours and fragrances as not the least, medicines. Plants have formed the basis of sophisticated traditional
medicinal systems among which are Siddha, Ayurvedic, Unani and Chinese amongst others. These systems of Medicine have given rise to some important drugs that are still in use even today. These Medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders and other herbal formulations (Balick and Cox, 1997; Samuelsson, 2004). The specific plants to be used and the methods of application for particular ailments were passed down through oral history. In more recent history, the use of plants as Medicines has involved the isolation and characterizations of pharmacologically active compounds from medicinal plants continue today.

Human body system is enriched with natural antioxidants and can prevent the onset as well as treat diseases caused and/or fostered due to free-radical mediated oxidative stress. Humans also take antioxidants through diet. In food, antioxidants are found in small quantities but capable of preventing or greatly retarding the oxidation of easily oxidizable materials (Tiwari, 2001). Many synthetic antioxidant compounds have shown toxic and/or mutagenic effects, which have stimulated the interest of many investigators to search natural antioxidant (Nagulendran et al., 2007). Even synthetic drugs used to treat various disorders are capable of producing free radicals which lead to oxidative stress and cause tissue damage. For example, non steroidal anti-inflammatory drugs (NSAIDs) are used widely in the treatment of pain, fever, inflammation, rheumatic and cardiovascular disease but chronic administration of these drugs lead to the generation of free radicals which may result in gastric erosions, gastric or duodenal ulceration and severe complications such as gastrointestinal haemorrhage and perforation (Kamboj, 2000). Recent studies have shown that plant extracts and its phytoconstituents have been found to be effective as antioxidants, radical scavengers and inhibitors of lipid peroxidation (Dash et al., 2007; Yildirim et
al., 2001; Ramchoun et al., 2009). The use of bioactive plant-derived compounds is on the rise, because the main preoccupation with the use of synthetic drugs is the side effects which can be even more dangerous than the diseases that they claim to cure. In contrast, plant derived Medicines are based upon the premise that they contain natural substances that can promote health and alleviate illness and prove to be safe, better patient tolerance, relatively less expensive and globally competitive. So, in respect of the healing power of plants, return to natural remedies is an absolute requirement of our time (Kamboj, 2000, Sen et al., 2009; Ramchoun et al., 2009). Therefore, Herbal Medicine is still the mainstay of about 75-80% of the world population and it is demand of time using drugs from plant sources or phytoconstituents to prevent and/or treat oxidative stress.

Seeds of *Semecarpus anacardium* (Marking nut) have been used in Indian traditional system of Medicines (Ayurveda and Siddha) either alone or as an ingredient of many polyherbal formulation for treating various ailments. *S. ancardium* of Anacardiaceae family is a medium sized tree grown in sub-Himalayan, tropical and central parts of India. Ayurveda describes it as a potent drug for neuritis, arthritis, leprosy helmintic infection and venereal disorders (Chopra et al., 1958; Sharma and Chaturvedi, 1965; Chattopadhyaya and Khare, 1969 and Singh et al., 2006). But supporting data are lacking. The nut milk extract of *Semecarpus anacardium* is one of the formulations of Siddha Medicine in India and this preparation is called as “Serankottai nei”. Cow’s milk and ghee are the main ingredients of this drug. The drug Serankottai nei was prepared following the procedure as given in the Formulary of Siddha Medicine (1972). Several pharmacological effects of this drug have been proved in our laboratory such as anti-oxidant, anti-inflammatory and anti-arthritis (Ramprasath et al., 2004,
2005, 2006a). Similarly anti-cancer (Mathivadhani et al., 2006, 2007a, 2007b) and anti-diabetic effects have also been investigated (Jaya et al., 2010). Apart from our investigation, some other investigators have also reported that various extracts of *Semecarpus anacardium* nuts such as aqueous and alcoholic extracts possessed to have anti-microbial and anti-bacterial activities (Mohanta et al., 2007; Nair and Bhide, 1996).
AIM AND SCOPE OF THE PRESENT INVESTIGATION

*Semecarpus anacardium* Linn, belongs to the family Anacardiaceae and its seeds have high priority and applicability in indigenous system of Medicine for treating various ailments. However, the mechanism of the pharmacological action of its nuts can be greatly established only by the isolation of its active principles and determination of structure and functional relationship. These informations encouraged us to isolate an active principle from *Semecarpus anacardium* nut milk extract. To our knowledge, there are no reports that detail the antioxidant activities of isolated compounds from *Semecarpus anacardium* nuts. Therefore, our goal is to isolate new active principles from *Semecarpus anacardium* nut milk extract and evaluate its antioxidant potentials in *in vitro* (cell free system). The assessment of the efficacy of each isolated compound has been made on the following basis.

- Primary Phytochemical screening
- Isolation and characterization of active principles
- Free radical scavenging activity (DPPH, Hydroxyl radicals and NO induced free radicals)
- Lipid peroxidation inhibition activity

This chapter deals with the above mentioned investigation.
MATERIALS AND METHODS
1.2 MATERIALS AND METHODS

1.2.1 Sources of chemicals

TLC aluminium sheet 20 x 20 Silica gel 60 F$_{254}$ obtained from Merck, Darmstadt Germany. Silica gel 60-120 mesh size for column chromatography, chloroform, methanol, diethyl ether, n-butanol, hexane, ethyl acetate, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemicals Co. (St. Louis, USA). Deoxyribose, nitric acid, sulphanilamide, naphthyl ethylene diamine, thiobarbituric acid (TBA), hydrogen peroxide (H$_2$O$_2$) and ascorbic acid were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. All other chemicals used were of analytical grade.

1.2.2 Formulation of the SA (Serankottai nei)

The drug Serankottai nei is a Siddha drug wherein Semecarpus anacardium (SA) and ghee are the main ingredients. The drug was prepared by boiling the nuts of SA (200g) with 500ml of cow milk. The decoction was recovered and the process was repeated three times. All the three portions of the milk nut decoction were mixed with ghee (1.5kg) and boiled till dehydrated and later it was filtered and stored.

1.2.3 Preparation of Extraction from Semecarpus anacardium nut milk extract

Thousand gram of Serrankottai nei (Semecarpus anacardium nut milk extract) was resoaked in 2.5 liter of methanol and kept in refrigerator for 3 days. Then, the filtrate was filtered through Whatmann filter paper No. 1 and this was repeated three to four times until the filtrate gave no coloration and concentrated
using vacuum rotary evaporator at 40°C. The methanolic concentrate was fractionated sequentially with petroleum, diethyl ether, chloroform and n-butanol. The n- butanolic fraction was evaporated to dryness. Before isolating an active principle from the n-butanolic concentrate, it was subjected to primary phytochemical screening to know its chemical constituents.

1.2.4 Preliminary Phytochemical Screening

The ethanolic extract of Serrankottai nei (Semecarpus anacardium nut milk extract) was subjected to preliminary Phytochemical screening of various plant constituents (Harborne, 1998; Kokate, 2001).

1.2.4.1 Test for Alkaloids

1.2.4.1.1 Wagner’s test

Wagner’s reagent: 1.2g of iodide and 2.0 g of potassium iodide were dissolved in 5 ml of Con. sulphuric acid and the solution was diluted to 100 ml with distilled water.

Procedure: Ten millilitres of extract was acidified by adding 1.5% v/v of hydrochloric acid and a few drops of Wagner’s reagent. Formation of yellow or brown precipitate confirmed the presence of alkaloid.

1.2.4.2 Test for Flavonoids

1.2.4.2.1 Shinoda’s test

Procedure: In a test tube containing 0.5 ml of extract, 5-10 drops of 1N hydrochloric acid and small piece of ZnCl were added and the solution was boiled for few minutes. In the presence of flavonoids, reddish pink colour was produced.
1.2.4.3 Test for Saponins

(i) Procedure: The extract was diluted with 20 ml of distilled water and it was agitated in a graduated cylinder for 15 minutes. The formation of 1 cm layer of foam shows the presence of saponins.

(ii) Procedure: One ml of the extract was treated with 1% lead acetate solution. Formation of white precipitate indicates the presence of saponins.

1.2.4.4 Test for Carbohydrates

A small quantity of the extract was dissolved separately in 4 ml of distilled water and filtered. The filtrate was subjected to Molisch’s test to detect the presence of carbohydrates.

Molisch’s Test

Procedure: Filtrate was treated with 2-3 drops of 1% alcoholic α-naphthal solution and 2 ml of Conc. $\text{H}_2\text{SO}_4$ was added along the sides of the test tube. Appearance of brown ring at the junction of two liquids shows the presence of carbohydrates.

1.2.4.5 Test for Phenols

(i) Ferric chloride test

Ten percent Ferric chloride: Ten gram of ferric chloride was dissolved in 100ml of distilled water.
Procedure: To 1ml of alcoholic solution of extract, 2ml of distilled water followed by drops of 10% aqueous ferric chloride solution was added. Formation of blue or green colour indicates the presence of phenols.

(ii) **Libermann’s test**

Procedure: A small quantity of alcoholic extract was dissolved in 0.5ml of sulphuric acid solution followed by the addition of a few drops of aqueous sodium nitrate solution. Formation of a red colour was obtained on dilution and it turned blue when made alkaline with aqueous sodium hydroxide solution indicates the presence of phenols.

1.2.4.6 **Test for Triterpenoids**

(i) **Libermann-Burchard test**

Procedure: Ten milligram of the extract was dissolved in 1 ml of chloroform; 1 ml of acetic anhydride was added following the addition of 2 ml of Conc. H₃SO₄. Formation of reddish violet colour indicates the presence of triterpenoids.

(ii) **Noller test**

Procedure: Five milligram of the extract was dissolved in 2 ml of 0.01% anhydrous stannic chloride in pure thionyl chloride. A purple colour formed, then changed to deep red after few minutes indicates the presence of triterpenoids.

1.2.4.7 **Test for Phytosterol**

The extract was refluxed with solution of alcoholic potassium hydroxide till complete saponification takes place. The mixture was diluted and extracted with
ether. The ether layer was evaporated and the residue was tested for the presence of phytosterol.

(i) **Libermann Burchard test**

Procedure: The residue was dissolved in few drops of diluted acetic acid, 3 ml of acetic anhydride was added followed by few drops of Conc. H$_2$SO$_4$. Appearance of bluish green colour shows the presence of phytosterol.

(ii) **Salkowski test**

Procedure: Ten milligram of the extract was dissolved in 1 ml of chloroform; 1 ml of Conc. H$_2$SO$_4$ was added carefully along the sides of the test tube. The red colour appeared at the junction of the two layers as a ring, indicating the presence of sterols.

1.2.4.8 **Test for Glycosides**

The extract was hydrolysed with HCl for few hours on a water bath and the hydrolysate was subjected to Legal’s or Borntrager’s test to detect the presence of glycosides.

(i) **Legal’s test**

Procedure: To the hydrolysate, add 1 ml of pyridine and a few drops of sodium nitroprusside solutions and then it was made alkaline with 0.1N Sodium hydroxide solution. Appearance of pink to red colour shows the presence of glycosides.
(ii) **Borntrager’s test**

Procedure: Hydrolysate was treated with chloroform and then the chloroform layer was separated. To this, equal quantity of dilute ammonia solution was added. Ammonia layer acquires pink colour that shows the presence of glycosides.

1.2.5 **Isolation and characterization of active principles from Serrankottai nei (Semecarpus anacardium nut extract)**

After knowing the chemical constituents from the extract of Serrankottai nei and it was checked on thin layer chromatography (TLC). This is the primary and fundamental step for isolating compounds either from natural sources or from chemicals. The n- butanolic concentrate was subjected to TLC using hexane and ethyl acetate in the ratio of 8:2 as a mobile phase in which four spots appeared (fig 1). The n-butanolic concentrate was chromatographed on silica gel column (Merk 60 -120 mesh, 750 g, 3.5 i.d. X 60 cm) and eluted successively with hexane and ethyl acetate (80: 20 ratio). A total of 50 fractions were collected at an interval of 10 ml each and monitored by thin layer chromatography (precoated silica gel merk-60 F254 0.25 mm thick plate). Fractions from 1-5 which formed pale green or straw yellow colour and showed single spot on TLC were pooled together in a clean vial and evaporated to dryness. Fractions from 15 -20 which formed as dark yellow colour showed single spot on TLC were pooled together in a clean vial and evaporated to dryness. Fractions from 25 -30 formed as light yellow colour showed single spot on TLC were pooled together in a clean vial and evaporated to dryness and finally fractions from 40 -50 which formed deep red colour and showed single spot on TLC were pooled together in a clean vial and evaporated to dryness. This process was repeated until getting satisfactory yield of each compound. The structure of the
Fig.1. Isolation of active principle from *Semecarpus anacardium* nut milk extract (Serrankottai nei) and seed extract
each compound was confirmed on the basis of IR, \(^1\)H NMR, \(^{13}\)C NMR and MS spectrum.

1.2.6 *in vitro* ANTIOXIDANT ACTIVITY (CELL FREE SYSTEMS)

1.2.6.1 Determination of DPPH Radical Scavenging Activity

The DPPH assay was performed as described by Koleva *et al.*, (2002).

Reagents

(i) DPPH (1, 1-diphenyl-2-picrylhydrazyl): Six milligram of DPPH was dissolved in 100 ml of absolute alcohol (Ethanol).

(ii) Methanol.

Procedure

About 10\(\mu l\) of each concentration (1.5-1000\(\mu g/ml\)) of test sample solution was added to 190\(\mu l\) of DPPH (150\(\mu M\)) in ethanol solution. After vortexing, the mixture was incubated for 20 minutes at 37\(^{\circ}\)C. The control solvent was without extract. The decrease in absorbance of test mixture (due to quenching of DPPH free radicals) was measured spectrophotometrically at 517nm and the percentage of inhibition was calculated. The IC\(_{50}\) values were determined as the concentration of the test mixture that gave 50% reduction absorbance from a control blank.

\[
\% \text{ of inhibition} = \frac{(\text{Control OD} - \text{Control blank}) - (\text{Sample OD} - \text{Sample blank})}{(\text{Control OD} - \text{Control blank})} \times 100
\]
1.2.6.2 Lipid Peroxidation Inhibition Assay

Lipid peroxide inhibition activity was estimated by Ohkawa et al., (1979).

Reagents

(i) Ten percent liver homogenate: Ten gm of liver tissue was homogenated with 100ml of KCl.

(ii) 0.15M Potassium chloride: 1.12 gm of potassium chloride was dissolved in 100 ml of distilled water.

(iii) 25µM Ferrous sulphate: 347.5 mg of ferrous sulphate was dissolved in 50 ml of distilled water.

(iv) 100µM Ascorbate: 880.65 mg of ascorbate was dissolved in 50 ml of distilled water.

(v) 10mM Potassium dihydrogen phosphate: Sixty eight mg of potassium dihydrogen phosphate was dissolved in 100 ml of distilled water.

(vi) 0.15mM KCl: One hundred and eighty eight mg of potassium chloride was dissolved in 100 ml of distilled of water.

(vii) 0.8% Thiobar butaric acid (TBA): Eight hundered mg of thiobarbituric acid was dissolved in 100 ml of distilled water.

Procedure

Ten percent liver homogenate was prepared using ice-cold KCl (0.15M) in a Teflon tissue homogenizer and the protein content was adjusted to 500µg/ml. In the control systems, 1ml of tissue homogenate, lipid peroxidation was initiated by
the addition of FeSO₄ (25µM), ascorbate (100µM) and KH₂PO₄ (10mM) and the volume was made up to 3ml with distilled water and incubated at 37°C for 30 min. In the test system, homogenate was incubated with different concentration of extracts (1.5-1000 µg/ml). The extent of inhibition of lipid peroxidation was evaluated by the estimation of thiobarbituric acid reactive substances (TBARS) level by measuring the absorbance at 532nm (Ohkawa et al., 1979). The percentage inhibition of lipid peroxidation was calculated by the formula,

\[
\text{Control OD} - \text{Test OD} \\
\% \text{ of Inhibition} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100
\]

1.2.6.3 Scavenging of Nitric Oxide Radical Activity

Nitric oxide radical scavenging assay was performed as described by Green et al., (1982).

Reagents

(i) 5mM Sodium nitroprusside: One hundred and forty nine mg of Sodium nitroprusside was dissolved in 100 ml of distilled water.

(ii) Phosphate buffer (pH 7.4):

Solution A: Sodium dihydrogen phosphate (NaH₂PO₄): 2.399 g of sodium dihydrogen phosphate was dissolved in 100 ml of distilled water.

Solution B: Disodium hydrogen phosphate (Na₂HPO₄): 2.839 gm of disodium hydrogen phosphate was dissolved in 100 ml of distilled water.
Nineteen ml of Solution A and 81 ml of Solution B were mixed and pH was adjusted to 7.4

(iii) Greiss reagent: One gram of sulphanilamide was dissolved in 2 ml of phosphoric acid and adds few ml of distilled water then added 100 mg of naphthylenediamine mixed and made up to 100 ml of distilled water.

Procedure

Aqueous sodium nitroprusside at physiological pH spontaneously generates nitric oxide (NO), which interacts with oxygen to produce nitrite, which can be estimated by using Greiss reagent. Sodium nitroprusside (5mM) in phosphate buffered saline was mixed with 3ml of different concentrations (1.5-1000µg/ml) of the extracts dissolved in methanol and incubated at 25ºc for 150minutes. The samples from the above were allowed to react with Greiss reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylenediamine was read at 546nm (Green et al, 1982). The experiments were repeated in triplicates. The percentage scavenging of nitric oxide radical activity was calculated by the following formula,

\[
\frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100
\]
1.2.6.4 Determination of Hydroxyl radicals Scavenging Activity

Hydroxyl radical scavenging activity was estimated by the method of Ohkawa et al., (1979)

Reagents

(i) 2.8 mM Deoxyribose: 37.5 mg of Deoxyribose was dissolved in 10 ml of Distilled water.
(ii) 0.1 mM Ferric Chloride: 16.2 mg of Ferric chloride was dissolved in 100 ml of distilled water.
(iii) 0.1 mM Ethylenediamine tetra acetic acid (EDTA): 3.72 mg of EDTA was dissolved in 100 ml of distilled water.
(iv) 0.1 mM Ascorbic acid: 1.76 mg of Ascorbic acid was dissolved in 100 ml of distilled water.
(v) 0.1 mM Hydrogen peroxide (H₂O₂): 113 µl H₂O₂ was mixed with 100 ml of distilled water.
(vi) 20 mM Potassium phosphate-KOH buffer (pH 7.4):

Solution A: One hundred and twelve mg of KOH was dissolved in 100 ml of distilled water.

Solution B: Two hundred and seventy two mg of KH₂O₄ was dissolved in 100 ml of distilled water.

Solutions A and B were mixed in equal volume.

Procedure

Hydroxyl radicals scavenging activity was determined by the estimation of competition between deoxyribose, catechol derivatives and crude extract sample (SA
nut milk extract) for the hydroxyl radicals generated from Fe$^{3+}$ Ascorbate-EDTA-
H$_2$O$_2$ system (Fenton’s reaction). The hydroxyl radicals attack deoxyribose, which
results in thiobarbituric acid reacting substance (TBARS). The reaction mixture
contained deoxyribose (2.8 mM); FeCl$_3$ 0.1 mM); K$_2$HPO$_4$- KOH buffer (20mM,pH
7.4); EDTA (0.1 mM); H$_2$O$_2$ (1.0 mM); Ascorbic acid (0.1 mM) and various
concentrations of catechol derivatives and crude extract (S.A nut milk extract) in a
final volume of 1 ml. The reaction mixture was incubated at 30°C for 60 minutes.
The formation of TBARS was read spectrometrically at 532 nm. The hydroxyl
radicals scavenging activity was determined by comparing absorbance of control
OD with treated OD.

\[
\text{Control OD} - \text{Test OD} \\
\% \text{ of Inhibition} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100
\]
RESULTS
1.3 RESULTS

1.3.1 PHYTOCHEMICAL SCREENING

Phytochemical screening of the extract revealed the presence of alkaloids, flavonoids, carbohydrates, phenols, steroids and glycosides which were represented in the Table 1.

**Table-1**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Test</th>
<th>Observation</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>Brown precipitate formed</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Flavonoids</td>
<td>Reddish pink colour developed</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Saponins</td>
<td>(i) 1 cm layer of foam was not formed</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) White precipitate was not formed</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Carbohydrates</td>
<td>Appearance of brown ring was formed</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Phenols</td>
<td>Formation of blue green colour</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Triterponoids</td>
<td>(i) Formation of reddish violet colour was not formed</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) A purple colour was not formed</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Phytosterol</td>
<td>(i) Bluish green colour was formed</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) Red colour was formed</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Glycosides</td>
<td>(i) Red colour was formed</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) Pink colour was formed</td>
<td></td>
</tr>
</tbody>
</table>

+ indicates presence, - indicates absence

1.3.1.1 Spectral data of catechol derivative I (Compound 1)

Figures (2-6) show the spectral values of the catechol derivative 1. IR 3455.81 (-OH) cm$^{-1}$, $^1$H NMR (300MHz CDCl$_3$) $\delta$ 0.808 (s, 3H, CH$_3$), 0.832 (s, 3H, CH$_3$), 1.193(s, 22H, CH$_2$), 1.948-1.92(m, 1H), 2.440-2.490 (m, 2H), 2.681(s, 3H, CH$_3$), 5.263(s, 2H, OH), 6.558 (s, 2H Ar-H ) ppm. $^{13}$C NMR (75.1MHz, CDCl$_3$) $\delta$ 13.88, 14.21, 22.78, 22.89, 25.75, 27.33 (CH), 29.10, 29.39, 29.57, 29.64, 29.78,
Spectroscopic Analysis of Catechol Derivative I

The compound I was obtained as a straw yellow colour and colloidal in nature. The molecular formula of this compound was determined from IR, TOFMS, $^1$H and $^{13}$C NMR. In the IR spectrum a peak at 3455.81 cm$^{-1}$ confirmed the presence of –OH group in the compound. In the $^1$H NMR spectrum a broad singlet peak at δ 5.26 ppm corresponds to the -OH group present in the compound. The aromatic protons resonated as a apparent singlet at δ 6.55 ppm. The aliphatic protons present in the compound appeared at δ 0.80-2.49 ppm. The methyl group present in the aromatic ring resonated as a singlet at δ 2.68 ppm. In the $^{13}$C NMR spectrum the two methyl groups present in the compound observed at δ 13.88 ppm and δ 14.21 ppm. The aromatic protons appeared at δ 120.32, 122.2, 128.08, 129.98, 142.05, 143.19. All the other aliphatic carbons observed at δ 22.78 –29.98 ppm. The methyl group present in the aromatic ring appeared at δ 31.90 ppm. Finally, the structure of catechol derivative I was confirmed by mass spectrum which showed a peak at m/z 349 corresponding to the empirical molecular formula C$_{23}$H$_{40}$O$_2$.

1.3.1.2 Spectral data of catechol derivative II (Compound 2)

Figures (7-11) show the spectral values of the catechol derivative II. IR 3467.13 (-OH) cm$^{-1}$, $^1$H NMR (300MHz,CDCl$_3$) δ 0.889(d, 6H, $J$=5.7Hz), 1.259(br, s, 14H, CH$_2$), 1.60 (br, s, 2H, CH$_2$), 2.023(br, s, 2H, CH$_2$), 2.645-2.579 (m, 2H, CH$_2$), 5.350(s, 2H, OH), 5.632 (br, s, 1H, CH), 6.730-6.650(m, 3H, Ar-H) ppm. $^{13}$C
NMR (75.1MHz, CDCl$_3$) δ 14.13 (CH$_3$), 22.82 (CH$_3$), 28.82 (CH$_2$), 27.26, 29.02, 29.32, 29.45, 29.50, 29.62, 29.73, 29.83, 31.82, 113.32 (Ar-CH) 121.61 (Ar-CH), 122.37 (Ar-CH), 125.79 (CH), 128.82 (Ar-CH), 130.39 (CH), 142.13 (Ar-OH), 143.54 (Ar -OH) ppm. TOF MS m/z 304.24

**Spectroscopic analysis of catechol derivative II**

The compound 2 was obtained as a dark yellow colour, colloidal in form. The molecular formula of this compound was determined on the basis of IR, TOFMS, $^1$H and $^{13}$C NMR. In the $^1$H NMR spectrum of the compound a peak was observed at δ 5.35 ppm which confirmed two –OH groups present in the compound. The aromatic protons resonated as multiplets in the region δ 6.65-6.73 ppm. The aliphatic protons present in the compound resonated at δ 0.88-2.64 ppm. In the $^{13}$C NMR spectrum the one methyl protons present in the compound appeared at δ 14.13 ppm. The aromatic protons appeared at δ 121.61, 122.37, 128.82. The other aliphatic protons appeared at δ 22.82, 25.68, 27.26, 29.02, 29.32, 29.45, 29.50, 29.62, 29.73, 29.83, and 31.82 ppm. Finally, the structure of catechol derivative II was confirmed by mass spectrum which showed a peak at m/z 304 corresponding to the molecular formula C$_{20}$H$_{32}$O$_2$.

**1.3.1.3 Spectral data of catechol derivative III (Compound 3)**

Figure (12-16) show spectral values of compound 3. R 3404.75 (-OH) cm$^{-1}$, $^1$H’NMR (300 MH$_z$, CDCl$_3$), δ 0.879 (t, 3H, J=6.75 Hz) 1.256 – 1.386 (br, s, 8H, CH$_2$), 2.018 (br, s, 2H, CH$_2$ ), 2.557-2.636 (m, 8H, CH$_2$), 2.772( s, 2H, CH$_2$) 5.354 (s, 2H, Ar- OH), 5.639 (br, s, 4H, Aliphatic alkene), 6.489-6.685(m, 3H, Ar-H) ppm.
Spectroscopic analysis of catechol derivative III

The compound 3 was obtained as light yellow colour and viscous in nature. In the \(^1\)H NMR spectrums the compound exhibited a broad singlet at \(\delta\) 5.35 ppm corresponding to the two –OH protons present in the compound. The aromatic protons resonated as an apparent multiplet at \(\delta\) 6.49 - 6.68 ppm. The aliphatic chain attached to the catechol ring was observed as multiplet at \(\delta\) 0.85-2.77 ppm. In the \(^{13}\)C NMR spectrum the carbons of the aromatic ring were observed at 112.95, 120.05, 121.07, 122.06, 142.20, 143.16, and aliphatic alkenes chain 127.9, 128.1, 129.9, 130.2 ppm. All the other aliphatic carbons resonated at 22.67-31.9 ppm. Finally the structure of catechol derivative III was confirmed by mass spectrum which showed a peak at \(m/z\) 317, corresponding to the molecular formula \(C_{21}H_{32}O_2\).

1.3.1.4 Spectral data of catechol derivative IV (Compound 4)

Figure (17-21) show spectral values of compound 4. \(^1\)H NMR (300MHz, CDCl\(_3\)) \(\delta\) 0.91 (d, 6H, \(J=7.2\) Hz), 1.305 – 1.255 (m, 30H, CH\(_2\)) 1.603 (s, 2H, CH\(_2\)), 1.996 (s, 1H, CH), 2.606 (S, 3H, CH\(_3\)), 2.786 – 2.751 (m, 2H, CH\(_2\)), 5.348 (s, 2H, OH), 6.827 – 6.681 (m, 2H, Ar-H) ppm. \(^{13}\)C NMR (75.1MHz, CDCl\(_3\)) \(\delta\) 14.13 (CH\(_3\)), 22.68, 22.71, 22.81, 25.66, 27.24, 29.00, 29.30,
The compound 4 was obtained as a deep red in colour and colloidal in nature. In the IR spectrum of the compound, a peak was observed at 3421.89 cm$^{-1}$ which indicate the presence of –OH group present in the compound. In the $^1$H NMR spectrum, the compound exhibited a broad singlet at $\delta$ 5.348 corresponding to the two–OH protons present in the compound. The aromatic protons were observed at $\delta$ 6.68 - 6.82 ppm. The aliphatic protons attached to the catechol ring were observed as a multiplet at $\delta$ 0.91- $\delta$ 1.99. The methyl group present in the aromatic ring was observed at $\delta$ 2.60 ppm. In the $^{13}$C NMR spectrum the aliphatic groups resonated at $\delta$ 14.13 ppm. All the other aliphatic carbons were observed at $\delta$ 22.68- 39.60 ppm. A peak at $\delta$ 31.95 ppm corresponding to the methyl group was present in the aromatic ring. The aromatic carbons resonated at $\delta$ 128.0, 128.1, 129.6, 129.9, 141.2, 143.2 ppm. Finally, the structure of catechol derivative IV was confirmed by mass spectrum which showed a peak at m/z 404, corresponding to the molecular formula C$_{27}$H$_{48}$O$_2$.

Yield of the compounds was varied from SA nut milk extract (a siddha prepared drug) and SA seeds which are shown in Table 2.
Table 2 Yield of compounds isolated from *Semecarpus anacardium* nut milk extract (a siddha preparation) and *Semicarpus anacardium* Seeds extract (extraction made directly from seeds)

<table>
<thead>
<tr>
<th>Isolated compounds</th>
<th>Total yield of catechol derivatives from SA nut milk extract (g/kg)</th>
<th>Total yield of catechol derivatives from SA seeds extract (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol derivatives I</td>
<td>1.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Catechol derivatives II</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Catechol derivatives III</td>
<td>0.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Catechol derivatives IV</td>
<td>1.2</td>
<td>2.4</td>
</tr>
</tbody>
</table>
ISOLATED COMPOUNDS

Catechol derivative I

Catechol derivative II

Catechol derivative III

Catechol derivative IV
1.3.2 *invitro* ANTIOXIDANT ACTIVITY OF ISOLATED CATECHOL DERIVATIVES FROM SA

1.3.2.1 1, 1-diphenyl-2-picrylhydrazyl (DPPH) Scavenging Activity

DPPH scavenging potential of isolated catechol derivatives and crude extract (*Semecarpus anacardium* nut milk extract) were shown in Table 3. DPPH scavenging activity of catechol derivatives and crude extract increased with increasing concentration from 30 to 1000µg. Catechol derivatives I and IV showed highest percent DPPH scavenging activities (86.57, 89.61 %), when compared to crude extract (SA nut milk extract) and catechol derivatives II, III (72.59, 79.53 and 84.05%) at 1000µg/ml.

1.3.2.2 Lipid Peroxidation Inhibition Activity

Lipid peroxidation inhibition activity of SA nut milk extract and its active principles catechol derivatives I-IV are given in table 4. The maximum percent lipid peroxidation inhibition activity was observed in catechol derivatives I and IV (74.65 and 76.04 %) compared to crude extract and catechol derivatives II and III (60.79, 67.67 and 76.04 %) at 1000µg/ml.

1.3.2.3 Nitric Oxide Scavenging Activity

Formation of nitric oxide by SNP in aqueous solution at physiological condition has been reported to occur by spontaneous oxidation. NO is a very unstable species, under aerobic condition it reacts with oxygen to produce intermediates such as NO₂, N₂O₄, N₃O₄ and a stable products such as NO₃⁻ and NO₂⁻. [NO radicals were determined indirectly with Griess reagent through the inhibition of nitrite production]. The nitric oxide antiradical activity of the catechol
derivatives and crude extract increased with increasing concentration which is represented in the Table 5. Both catechol derivatives and crude extract showed potent nitric oxide scavenging activity in a concentration dependent manner. The Highest percent inhibition of nitric oxide was observed in catechol derivative I (77.06 %) and catechol derivative IV (80.80 %) compared to crude extract (66.71 %) catechol derivative II and III (69.20, 71.50 %). at 1000µg/ml.

1.3.2.4 Hydroxyl Radical Scavenging Activity

Hydroxyl radical is the principal contributor for tissue injury. The formation of Hydroxyl radical from Fenton reaction was quantified using 2, deoxy-D-ribose degradation. The catechol derivatives and crude extract were found to exhibit a concentration dependent hydroxyl radical scavenging activities indicated as percentage inhibition in Table 6. Hydroxyl radical scavenging activity was found to be increased in catechol derivative I (75.57%) and catechol derivative IV (77.60%) when compared to crude extract (61.59%) catechol derivative II (69.84 %) and catechol derivative III (74.95) at 1000µg/ml.
DISCUSSION
1.4 DISCUSSION

1.4.1 DPPH Scavenging Activity

DPPH radicals have been used extensively as stable radicals to preliminarily evaluate the antioxidant activities of various compounds as well as plant extracts. In the DPPH test, the catechol derivatives (I-IV) and SA nut milk extracts were able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction. In the present study, Catechol derivatives I and IV showed highest radical scavenging activity. From this point of view, it can be concluded that catechol derivatives are potent in proton donating ability on DPPH to produce DPPHH which is important mechanism of antioxidants. These results corroborate with the findings of many research groups, who reported such correlations between total phenolic content and free-radical scavenging activity (Ardestani & Yazdanparast, 2007).

1.4.2 Lipid peroxidation inhibition activity

Lipid peroxidation has been implicated in the pathogenesis of various diseases. It is well established that bioenzymes are very much susceptible to LPO, which is considered to be the starting point of many toxic as well as degenerative processes. In the present study, inhibition of lipid peroxidation induced by FeSO₄ in liver homogenate and the degree of lipid peroxidation was assayed by estimating the thiobarbituric acid-reactive substances (TBARS) by using the standard method.
(Okhawa et al., 1979). The catechol derivatives and crude extract with liver homogenate undergo rapid peroxidation when incubated with FeSO₄ and produce peroxide and they attack the biological materials (Aruma, 1996). This leads to formation of Malondialdehyde (MDA), a product of lipid peroxidation and other aldehydes which form pink chromogen with TBA, absorbing at 535nm (Kosugi et al., 1987). During a hepatic damage condition, the levels of MDA would have been increased. Therefore, the increase in the MDA level in liver homogenate indicates the increase in the lipid peroxidation (Trible et al., 1987). Upon combining incubation of liver homogenate with SA nut milk extract and its constituents catechol derivative I-IV decreased the levels of MDA. Antioxidant may often resist the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by many other mechanisms (Youdim et al., 2001). Our results were consistent with findings of Kappus et al., (1977) who reported that catechol possesses anti-peroxidative effect in the liver of CCL₄ induced rat.

1.4.3 Nitric oxide scavenging activity

Nitric oxide is a free radical, produced in mammalian cell involved in the regulation of various physiological processes. However, excess production of nitric oxide has been implicated in various inflammatory and degenerative diseases (Ray et al., 2002). Inflammatory cells, which are components of atherosclerotic plaques, produced extracellular NO which presumably interfered with the endothelial cell proliferation (Marcocci et al., 1994). Thus, the pathophysiology of the endothelium was modulated by NO. In the present study, the investigation of NO scavenging activity was based on the release of NO from sodium nitroprusside in physiological solution and determined with Griess reagent through the inhibition of nitrite production. The decreased level of nitrite in the reaction indicated an increased scavenging effect against NO since the amounts of nitrite were directly
proportional to the amounts of NO radicals in the reaction mixture. These results indicate that catechol derivatives and crude extract have scavenged NO in the reaction, which might influence the protection of cells, especially endothelial cells, by neutralizing the NO produced in oxidative stress.

1.4.4 Hydroxyl radicals scavenging activity

Hydroxyl radicals are considered to be one of the rapid initiators of lipid peroxidation process, abstracting hydrogen atoms from polyunsaturated fatty acid which brings about peroxidic reactions of membrane lipids and also from each of the carbon atom of the sugar moiety of DNA causing oxidative damage to DNA (Kitada et al., 1979). These effects have been implicated in mutagenesis, carcinogenesis and aging (Halliwell et al., 1999). Ferric-EDTA incubated with H₂O₂ and ascorbic acid at pH 7.4, produces hydroxyl radicals and was detected by their ability to degrade 2-deoxyribose into fragments, on heating with TBA at low pH forming a pink chromogen (Halliwell et al., 1987, Aruoma et al., 1989). The maximum activity was observed in catechol derivative I and IV, followed by crude extract and catechol derivatives II and III. Catechol derivatives have caused the removal of hydroxyl radical and prevented the degradation of 2-deoxyribose. From this result, it can be concluded that catechol derivatives are powerful scavengers of 'OH radical and therefore prevent 'OH radical related pathophysiological diseases.
SUMMARY & CONCLUSION
1.5 SUMMARY AND CONCLUSION

Free radicals are chemical species containing one or more unpaired electrons, like hydrogen atom, most transition metal ions, nitric oxide and oxygen, with two unpaired electrons. In living systems, free radicals are constantly generated and they can cause extensive damage to tissues and biomolecules, leading to various disease conditions, especially degenerative diseases and extensive lysis. The most effective way to eliminate free radicals which cause oxidative stress is with the help of antioxidants. Antioxidants prevent free radical-induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition.

Many synthetic drugs protect against oxidative damage, but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional Medicines. In almost all the traditional systems of Medicine, medicinal plants play a major role and constitute their backbone. Medicinal plants are the most important source of life saving drugs for a majority of the world’s population.

One such medicinal plant is Semecarpus anacardium (Bhallataka) have been used for medicinal and non medicinal purpose since ancient times. The toxicity of S.A. precludes its mega scale production particularly as a Medicine at industrial level. However, the traditional healer and physicians of Indian system of Medicine continue to use Semecarpus anacardium nut in various forms in their clinical practice. Semecarpus anacardium nut milk extract (Serrenkottai nei) is a Siddha Medicine and used to treat various ailments. In our laboratory, several investigations have been undertaken to test the efficacy of Serrenkottai nei in in vitro and in vivo models. Not many studies have been done in connection with isolation and
characterization of active principles either from Serrenkottai nei (Siddha Medicine) or *Semecarpus anacardium* seeds. Hence, we isolated four structurally similar catechol derivatives from “Serrenkottai nei. The antioxidant potential of both isolated compounds and crude SA nut milk extract were assessed in *in vitro* (cell free system) to get better insight in to the activity of these compounds and crude extract, comprehend their narrow therapeutic margin. It is also important to understand the Siddha inspired investigation of this traditionally acclaimed medicinal plant. The isolated catechol derivatives and SA nut milk extract extracts were tested for their radical quenching ability against a sequence of radicals, namely DPPH, H$_2$O$_2$, NO, LPO and reducing power.

The four catechol derivatives and SA nut milk extract effectively scavenged or inhibited all the radicals tested. Among the four catechol derivatives, catechol derivatives I and IV were the most effective radical scavenger, followed by SA nut milk crude extract and catechol derivatives II and III. In general, antioxidant and radicals scavenging activities are dependent on structure and substitution pattern of hydroxyl group. Our results showed that catechol derivatives I and IV exhibited effective antioxidant and radical scavenging activity *in vitro*, due to the presence of dihydroxyl and a methyl group in the aromatic ring and absence of double bond in the aliphatic side chain. This may be the reason for more effectiveness of these 2 drugs. But in the case of catechol derivatives II and III, less activity was observed which may be due to the absence of methyl group in aromatic ring and presence of double bond in the aliphatic side chain. Based on these results, it may be concluded that these catechol derivatives might be implying their use in pharmacological and food industries due to its antioxidant properties.
Conclusive remark

Both *Semecarpus anacardium* nut milk extract (Serrankottai nei) and *Semecarpus anacardium* nut extract (extraction made directly from SA nut) have been checked on thin layer chromatography (TLC) to know the variation and chemical components (active principles) present in them. Both the extracts showed four compounds (4 spots) on TLC in the same mobile phase Hexane and ethylacetate in the ratio of 8:2 (Fig 1 and 2). But yield of the compounds from each extract showed much variation. *Semecarpus anacardium* nut milk extract yielded 3gm/1000gm and *Semecarpus anacardium* nut extract (direct) yielded 6gm/1000gm (Table 2). The yield of the compounds obtained from SA nut milk extract was less when compared to extraction made directly with SA nut. The reason is, SA nut milk extract was mostly made up of milk and ghee. The ingredients milk and ghee have been washed away by subsequent fractions of organic solvent. Therefore, to isolate a compound from SA nut milk extract was not desirable, but, to isolate a compound from SA nut extract was desirable due to its high yield of compound (active principles). Moreover, from the results, the safety and beneficial effects of these derivatives can’t be concluded. Therefore, further animal experimental studies are needed to check the beneficial and adverse effect in short term and long term usage of this drug. Hence, the next chapter deals with acute and sub acute toxicity studies of the active compounds in rats.
REFERENCES
1.6 REFERENCES


Green LC, Wagner DA, Glogowaki J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate and in biological fluids. Anal Biochem.1982; 126:131.


Kamboj VP, Herbal medicine, Current Science, 78, 2000, 35-39


Mathivadhani P, Shanthi P, Sachdanandam P. Hypoxia and its downstream targets in DMBA


Nair A, Bhide SV. Sntimicrobial properties different parts of semecarpus anacardium. Indian drugs. 1996; 33:323-328.


