2. REVIEW OF LITERATURE

2.1 General

Prior to any research work, systematic literature review is crucial as it reveals the nature of the work previously carried out in the particular field, the methods adopted, its successfulness and the recent strides in the field. The present work is aimed at the isolation of compounds from various extracts of *E. crassipes* and its biological and pharmacological activities. Hence, an extensive search of literature pertinent to phytochemical aspects of *E. crassipes*, biological and pharmacological facet of plants with special emphasis on antibacterial, antifungal, antioxidant, acute oral toxicity, wound healing activity of extracts and polyherbal formulation, skin cream testing and larvicidal, pupicidal activity of other plant extracts and in particular *E. crassipes* for a period of 63 years (1949-2012). A total of 800 papers were reviewed of which around 102 papers describes the work carried out with *E. crassipes*.

2.2 *Eichhornia crassipes* (Mart.) Solms

Waterhyacinth (*Eichhornia crassipes* (Mart.) Solms) is an herbal product (**Quayum, 2007**) rich in phytochemicals (**Bakr *et al,* 1984; Virabalin *et al,* 1993; Greca *et al,* 1991; Greca *et al,* 1992a**).

2.2.1 Taxonomy

The English common names of *E. crassipes* (family *Pontederiaceae*; sub-family *Trollioideae*; tribe *Eichhornieae* (**Center *et al,* 2002**) are waterhyacinth, water hyacinth and water-hyacinth. 'Waterhyacinth' is the standardized spelling adopted by the Weed Science Society of America (**WSSA, 1984**) to denote that it is not an aquatic relative of true "hyacinth" (*Hyacinthus* spp.), as the two-word spelling suggests. The plant will be mentioned with its Botanical name *E. crassipes* in the following chapters.

2.2.2 Origin, geographical distribution, growth and reproduction of the plant

E. crassipes, a member of the pickerelweed family (Pontederiaceae) (**Agunbiade *et al,* 2009**) is indigenous to South America, particularly to the Amazonian basin (**Mitchell *et al,* 1976**). The plant has invaded freshwater systems in over 50 countries and, according to recent climate change models, its distribution may expand into higher latitudes as temperatures rise. *E. crassipes* is especially pervasive throughout Southeast Asia, the

South Eastern United States, Central and Western Africa and Central America (**Villamagna and Murphy, 2010**).

E. crassipes grows in all types of freshwater, lentic and lotic. The plant might be exceptionally productive since it is a warm water species with submerged roots and aerial leaves like emergent macrophytes (**Westlake, 1963**). Plant growth is described in two ways either by reporting the percentage of water surface covered in a period of time or by reporting the plant density in units of wet plant mass per unit of surface area (**EPA, 1988**). So, under normal conditions, loosely packed waterhyacinth can cover the water surface at relatively low plant density (10 kg/m^2 wet weight) and can reach maximum density of 50 kg/m^2 wet weight before growth ceases (**Reddy and Sutton, 1984**).

Growth of *E. crassipes* depends on various ecological factors. The chief limiting factors for regular plant growth and development are the ability of the plant to use solar energy, the nutrient composition of the water, cultural methods, environmental factors (**Nesic and Jovanovic, 1996**) salinity, temperature, nutrients, disturbance, natural enemies (**Wilson *et al,* 2001**) and pH levels (**Malik, 2007**). The plant can tolerate salinity up to 1.3-1.9 ppt and even below 1 ppt (**Chakrabarthy, 2009**) and grows fast at temperatures from 20 to 30 °C, but growth fully stops at temperatures from 8 to 15 °C (**Stephenson *et al,* 1980**). It can withstand near freezing temperature (<5°C) for a limited period, but exhibits a steady decline in regrowth potential (**Owens and Madsen, 1995**). Efficient intake of calcium, magnesium, sulfur, iron, manganese, aluminum, boron, copper, molybdenum, zinc, nitrogen, phosphorus and potassium favors its growth over other aquatic species (**Shanab *et al,* 2010**) eventhough nitrogen and phosphorus levels are considered as the most important limiting factors.

E. crassipes reproduces in both a generative and vegetative way. It commonly forms dense, interlocking mats due to its rapid reproductive rate and complex root structure (**Mitchell, 1985**).

2.2.3 Contemporary scenario of waterhyacinth

The rapid spread of the plant leads to the formation of mat-like coverage of fresh water ways. The effect of waterhyacinth on phytoplankton, zooplankton, macroinvertebrates, fish, and birds is deleterious (**Villamagna and Murphy, 2010**). Skin rash, cough, malaria, encephalitis, bilharzia, gastro intestinal disorders and

schistosomiasis are common among the people near water bodies infested by *E. crassipes*. Socio-economic impacts of *E. crassipes* are multi-faceted as it greatly affects fishing, boating, irrigation, municipal water supply (**Opande *et al,* 2004**) and navigation (**Malik, 2007**). Control measures taken to prevent the growth of waterhyacinth are difficult and hence measures are taken to minimize the plant density to the extent that economic and environmental effects are reduced. The mechanical, physical and chemical methods used to control *E. crassipes* have their own advantages and constraints and even more long term results are not obtained in any of the methods. The cost incurred with these control measures is also enormous. However, *E. crassipes* can be harvested mechanically and used in a way beneficial to society. Although *E. crassipes* is considered as the world's worst aquatic weed, there are areas wherein this plant finds enormous uses and some areas remain unexplored.

2.2.4 Phytochemicals in *E. crassipes*

E. crassipes possesses nutritionally important compounds like phenolics, flavonoids, glutathione (**Malik, 2007**), and many other metabolites. The chemical composition of *E. crassipes* from different geographic regions is found to vary (**Chakrabarthy, 2009**). The protein (**Chakrabarthy, 2009**), hemicellulose, cellulose, lignin, and magnesium contents in *E. crassipes* are documented (**Gunnarsson and Peterson, 2007**).

2.2.4.1 Phenolic compounds

Phenolic compounds are a large and diverse group of molecules, which include many different families of aromatic secondary metabolites in plants. Specialised phenol cells in *E. crassipes* (**Ahmed *et al,* 1982**) contain *p*-hydroxybenzoic, ferulic, chlorogenic, protocatechuic, vanillic, *p*-coumaric (**Martyn and Cody, 1983**), and o-hydroxy phenols (**Martyn *et al,* 1983**). Phenolic compounds are detected in the leaves (**Anjana and Matai, 1990; Center and Wright, 1991**), methanol extract (**Vasu *et al,* 2009; Shanab *et al,* 2010**) and aqueous extract (**Lata and Dubey, 2010a**) of *E. crassipes*. 4-Methylresorcinol, 2-methylresorcinol, catechol, pyrogallol, and genetisic, *p*-hydroxybenzoic, syringic, vanillic and salicylic acids have been identified by TLC in the ethanolic shoot extract, whereas 4-methylresorcinol, 2-methylresorcinol, resorcinol, catechol, and genetisic and salicylic acids were reported in rhizome of *E. crassipes* (**Lata *et al,* 2010b**). 1(2, 4-Dihydroxyphenyl) 2(4-methoxy-3-nitrophenyl) ethanone was identified in the ethanol extract by GC-MS (**Muthunarayanan *et al,* 2011**). The total polyphenol content

of the aqueous and methanol extract of *E. crassipes* has been reported to be 90.12 ± 7.26 and 184 ± 19.12 (µg CE/mg) respectively (**Ho *et al,* 2012**).

A benzoindonic compound, 2,5-dimethoxy-4-phenyl-benzoindenone (List 1.**1**) , a red oily metabolite isolated from *E. crassipes*, inhibits the growth of the fungus *Candida albicans* (**Greca *et al,* 1991**). Phenalene metabolites (List 1. **2-5**) (**Greca *et al,* 1992a**) and permethylated derivatives of ten aromatic metabolites with the phenalene skeleton (List 1. **2-11**) (**Greca *et al,* 1992b**) were isolated from the acidic and neutral portion of ethyl acetate extract of *E. crassipes*. Compounds **2-5** inhibited the growth of the freshwater alga *Porphyridium aerugineum* (**Greca *et al,* 1992a**).

2-Hydroxy-8-(4-hydroxyphenyl)-phenalen-1-one (List 1. **12**) and 2-hydroxy-8-(3,4-dihydroxyphenyl)-phenalen-1-one (List 1. **13**) have been isolated from the acetone extract of roots and leaves of *E. crassipes* (**Hölscher and Schneider, 2005**). The structures of certain phenalene compounds isolated from *E. crassipes* by **Greca *et al*** were revised based on 2D-NMR analysis and found to be 4,8,9-trimethoxy-1-phenyl-2,3-dihydro-1H-phenalene, 4,8,9-trimethoxy-1-(4′methoxyphenyl)- 2,3-dihydro-1H-phenalene,4,4′′,8,8′′ ,9,9′′-hexamethoxy-1,1′′-diphenyl-2,2′′,3,3′′-tetrahydro-7,7′′-bi(1H-phenalene),and 6,6′′,8,8′′,9,9′′,4′,4′′′-octamethoxy-1,1′′diphenyl-2,2′′,3,3′′-tetrahydro-7,7′′-bi(1H-phenalene) (List 1. **14-18**) (**Greca *et al,* 2008**). Compound **2**, and compounds **19-21** (List 1) were isolated from the ethyl acetate extract by column chromatography (**Greca *et al,* 2009**). Six new phenylphenalenes (List 1. **22-27**) and several known compounds (List 1. **28-31**) were isolated from the ethyl acetate fraction of the methanol extract of waterhyacinth (**Wang *et al,* 2011**). The phenalenes, sterols and dimethylesters isolated are represented in List 1.

2.2.4.2 Flavonoids

The presence of flavonoids in *E. crassipes* (**Lata and Dubey, 2010c; Viado, 2006**) makes it an attractive source of antioxidants. The flavonoid and flavonol content of water extract and methanol extract of *E. crassipes* was estimated as 9.97 ± 0.23 (µg RE/mg), 1.14 ± 0.01 (µg CE/mg), 25.60 ± 6.30 (µg RE/mg) and 3.08 ± 0.06 (µg CE/mg) respectively (**Ho *et al,* 2012**). The presence of apigenin, tricin, chrysoeiol, azaeleatin, gossypetin and luteolin in the shoot and rhizome of the plant was identified by paper chromatography (**Nyananyo *et al,* 2007**) and TLC (**Lata and Dubey, 2010c**) whereas kaempferol, orientin (**Nyananyo *et al,* 2007**), quercetin, isovitexin, and kaya flavone (**Lata**

and Dubey, 2010c) are also present in the shoot and rhizome. The ethanol extract contains 4H-pyran4-one,2,3-dihydro-3,5-dihydroxy-6-methyl (**Muthunarayanan *et al,* 2011**). From the purple flowers, [6-*m*-*O*-{delphinidin 3-*O*-(6 *m*-*O*-(β-D-glucopyranosyl)-β-D-glucopyranosyl)}], and [6-*m*-*O*-{apigenin 7-*O*-(β-D-gluco-pyranosyl)}] malonate were extracted with 5% acetic acid (**Toki *et al,* 1994**).

2.2.4.3 Tannins

Tannins are present in the methanol and aqueous extracts of *E. crassipes* (**Vasu, *et al,* 2009; Lata and Dubey, 2010a; Viado, 2006**).The plant detritus and decayed materials after cutting or herbicide application release tannin-like compounds (**Ahmed *et al,* 1982**).

2.2.4.4 Alkaloids

Phytochemical investigation of the plant showed the presence of alkaloids in *E. crassipes* (**Lata and Dubey, 2010a; Vasu, *et al,* 2009; Ndubuisi *et al,* 2007**). Qualitative separation of alkaloids from *E. crassipes* by TLC revealed that cytisine and tomatine are present both in the shoot and rhizome, whereas codeine, thebaine and quinine are present in the shoot, and the rhizome contains nicotine (**Lata and Dubey, 2010d**). GC-MS analysis of the methanol extract of *E. crassipes* showed the presence of 18,19-secoyohimban-19-oic acid, and 16,17, 20, 21-tetradehydro-16-(hydroxymethyl)-methyl ester (**Shanab *et al,* 2010; Shanab *et al,* 2011**), whereas pipradrol, and 1H-pyrrole,1-phenyl were detected in the ethanol extract (**Muthunarayanan *et al,* 2011**).

2.2.4.5 Terpenoids

Terpenoids were detected in various extracts of *E. crassipes* (**Shanab *et al,* 2010; Vasu *et al,* 2009; Lata and Dubey, 2010a**). 3,7,11,15-Tetramethyl-2-hexadecen-1-ol and phytol were identified in the ethanol extract by GC-MS (**Muthunarayanan *et al,* 2011**). Growth regulating substances, indole compounds and gibberellins were separated from the roots of the plant (**Sircar and Chakraverty, 1962; Sircar and Ray, 1961**). Carotene was extracted from *E. crassipes* by different methods. (**Neogi and Rajagopal, 1949**; **Panchanadikar *et al,* 2005**).

2.2.4.6 Sterols

Sterols are present in various extracts of *E. crassipes* (**Vasu *et al,* 2009; Lata and Dubey, 2010a**). From the ethyl acetate extract, 4α-methyl-5α-ergosta-8,14,24(28)-triene-

3β,4β-diol (List 1. **32**), 4α-methyl-5α-ergosta-8,24(28)-diene-3β,4β-diol (List 1. **33**), and 4α-methyl-5α-ergosta-7,24(28)-diene-3β,4β-diol (List 1. **34**) were isolated (**Greca *et al,* 1991**), whereas campesterol**,** stigmasterol and β-sitosterol were detected in the sterol mixture isolated from the acetone extract (**Goswami *et al,* 1983**). 6α-Hydroxystigmata-4, 22-dien-3-one **(**List 1. **35**), a novel steroid, has also been isolated (**Wu *et al,* 1991**).

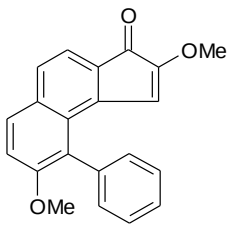
2.2.4.7 Glycosides

Glycosides (**Ndubuisi *et al,* 2007**), in particular cardiac glycosides (**Lata and Dubey, 2010**), were reported in the chloroform and aqueous extracts of the shoot of *E. crassipes*, respectively. Monogalactosyldiglycerides and digalactosyl-diglycerides are the major glycolipids. Phospholipids found in the roots, leaf stalks and flowers are respectively phosphatidylcholine, phosphatidylglycerol and phosphatidylethanolamine. The major fatty acids in the roots, leaf stalks, leaves and flowers are palmitic and linoleic, linoleic, palmitic, linolenic and linoleic, respectively (**Lakshminarayana *et al,* 1984)**. Stigmatic exudates of *E. crassipes* contain the soluble sugars, fructose, sucrose, and free fatty acids (**Kandasamy and Vivekanandan, 1983**). Analysis of the polysaccharide revealed that the heteropolysaccharide of *E. crassipes* is composed of D-xylose, L-galactose and L-arabinose (**Anjaneyalu *et al,* 1983**).

A galactomannan and a branched (1→3)-β-D-glucan were isolated from *E. crassipes*. The galactomannan, purified from the cold water extract, is composed of D-galactose and D-mannose in a ratio of 1.0:2.8. It has a (1→4)-linked D-mannose backbone, with one out of three D-mannose residues being substituted with a single α-D-galactosyl unit. The branched (1→3)-β-D-glucan isolated from the hot water extract has a main chain composed of β-(1→3)-linked D-glucopyranosyl residues, and two single β (1→6)-D-glucopy-ranosyl groups attached as side chains to, on an average, every 5 sugar units of the main chain. In addition, the branching of the β-glucan occurs regularly at O-6 of the β-(1→3)-linked backbone (**Issa, 1988**). In the water soluble sugars isolated from *E. crassipes*, the predominant ones were galactose, glucose, xylose and arabinose (**Arifkhodzhaev and Shoyakubov, 1995**). Sulfated polysaccharides were extracted from the root, rhizome, petiole and leaf of *E. crassipes* and the sulphate content was found to be rich in the petiole than the other plant parts. Proximate composition of *E. crassipes* portions

showed that all parts displayed low lipid and nitrogen contents. Moisture values ranged from 86 (leaves) to 93 (petiole and rhizome), with no significant differences ($p > 0.05$) among replicates. Monosaccharide compositions from *E. crassipes* showed the presence of galactose, glucose, arabinose, xylose in all parts, mannose and xylose in the root and rhizome of the plant (**Santos *et al,* 2012**).

α-Cellulose present in *E. crassipes* (**Gaonkar and Kulkarni, 1986; Gaonkar and Kulkarni, 1987; Ismail and Naby, 1995**) was etherified with chloroacetic acid in a solvent medium giving carboxymethyl cellulose (**Barai *et al,* 1997**). Enzymatic hydrolysis of *E. crassipes* yielded 10.7 mg of reducing sugars per 100 mg pretreated biomass in a 24 h reaction. Roots contain large amounts of polysaccharides, such as cellulose and hemicellulose (**Mishima *et al,* 2006**). Treatment of shoot and root biomass of *E. crassipes* with NaOH and CS_2 yielded the alkali-treated straw and cellulose xanthogenate, respectively. Alkali treatment removes most of the lignin and hemicellulose from the raw plant material, whereas the formation of cellulose xanthogenate introduces new C=S and O–CS–S functional groups (**Zhou *et al,* 2009**).



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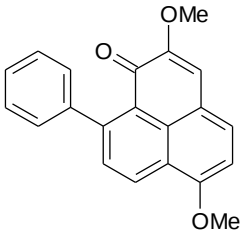

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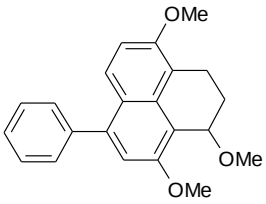

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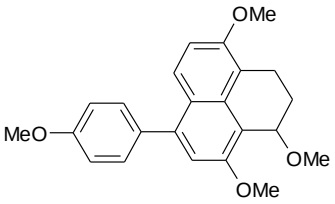

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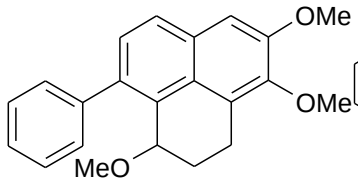

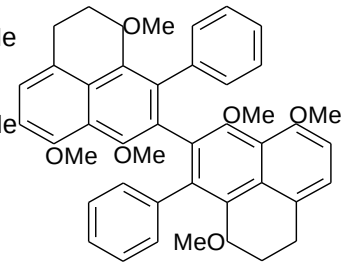

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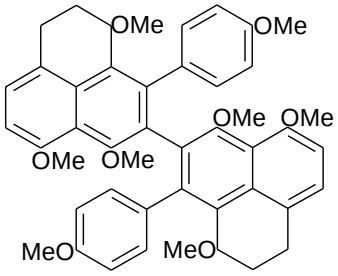

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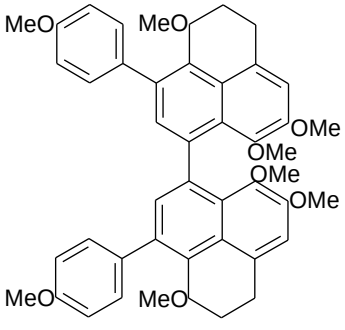

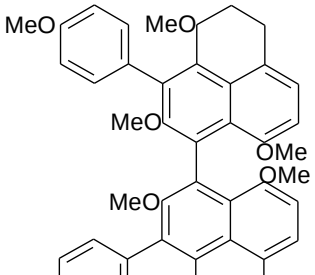

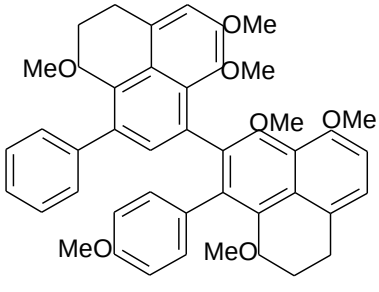

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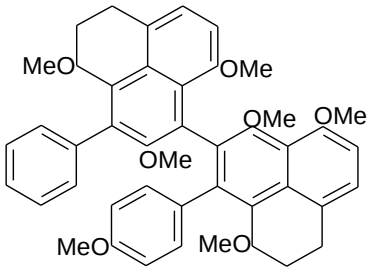

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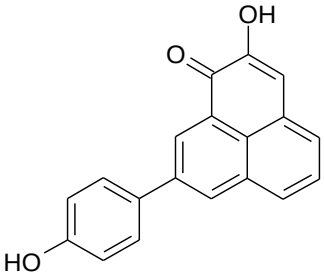

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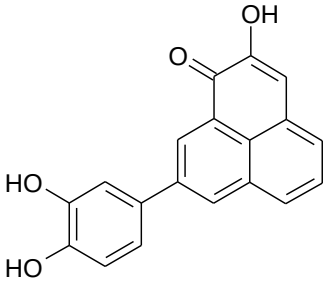

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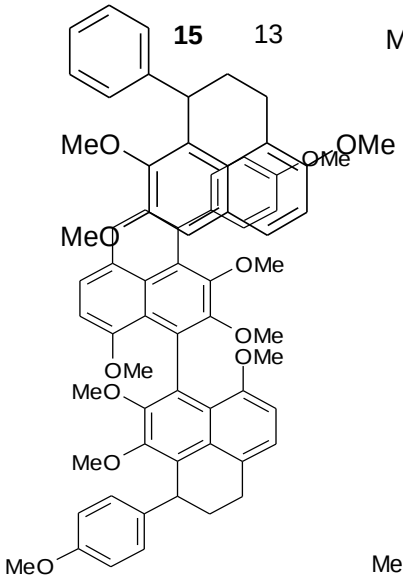

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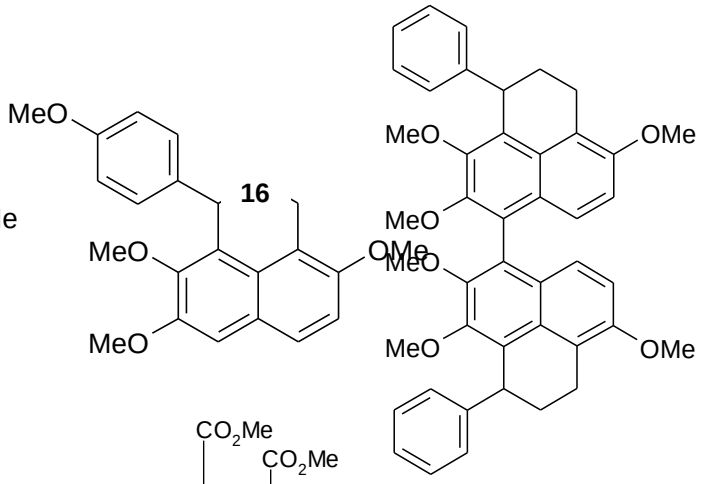

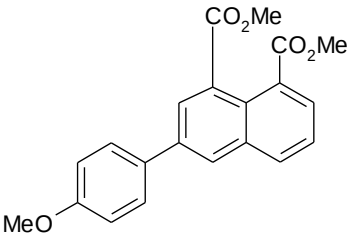

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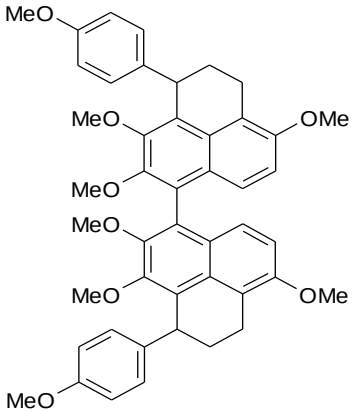

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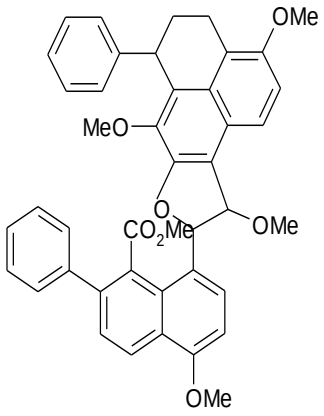

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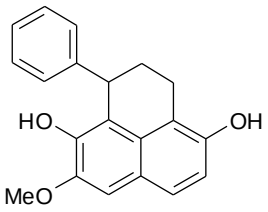

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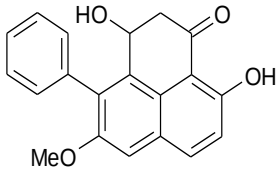

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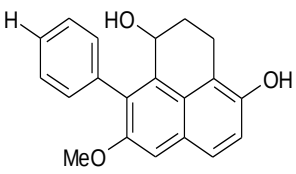

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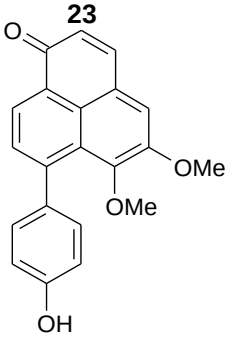

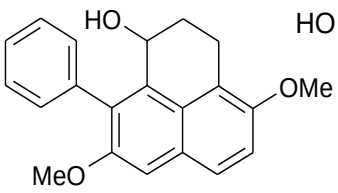

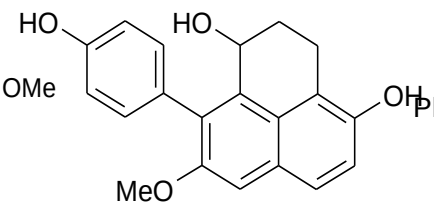

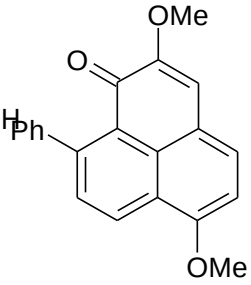

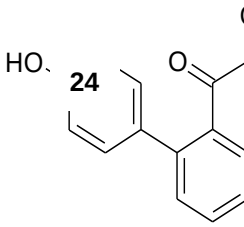

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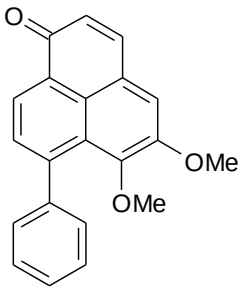

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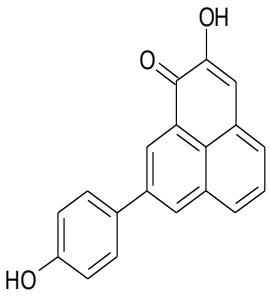

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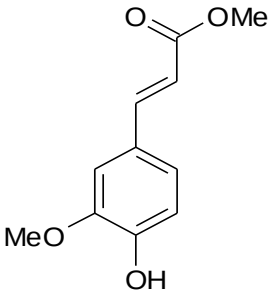

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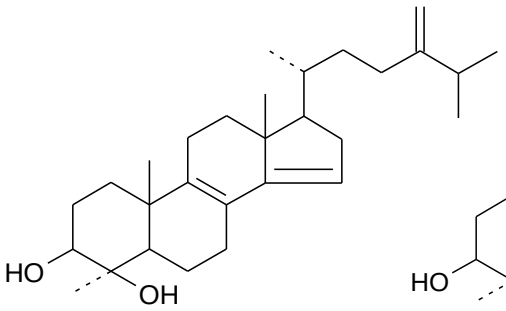

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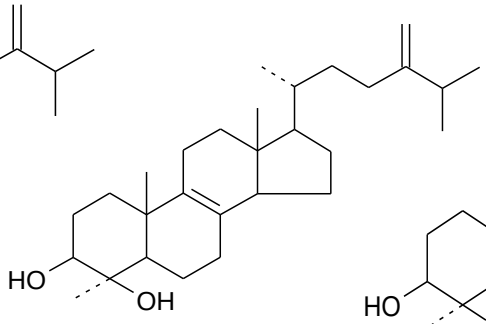

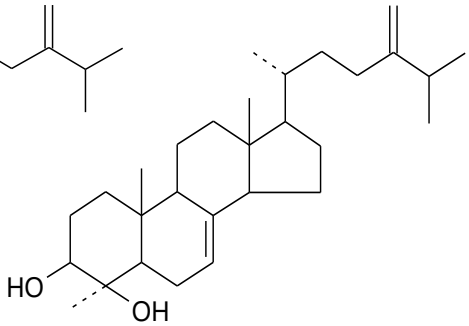

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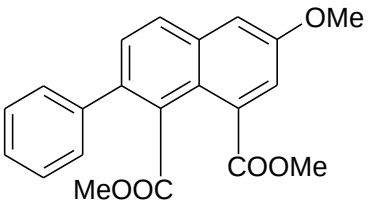

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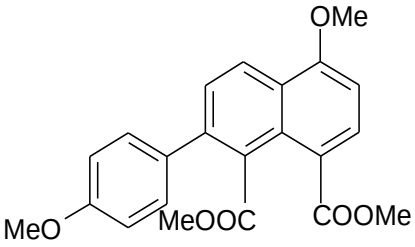

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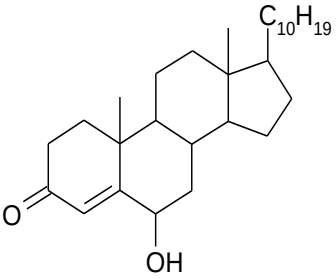

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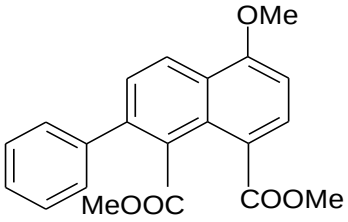

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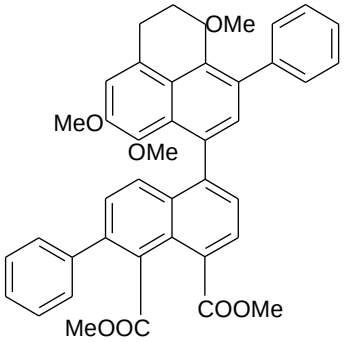

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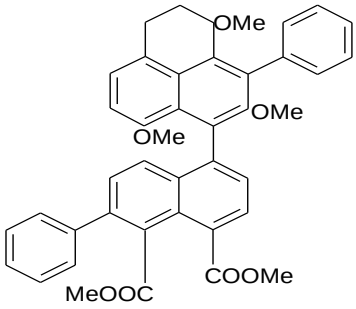

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List 1. **1**, *1,2,5-dimethoxy-4- phenyl- benzoindenone;* **2**, *2,6-dimethoxy-9-phenyl-1H-phenalen-1-one (lachnanthtocarpone red dimethyl ether);* **3**, *4,9-dimethoxy-7-phenyl-2,3-dihydrophenalen-1-olo-O-methyl ether*; **4**, *4,9-dimethoxy-7-(4'-methoxy-phenyl)-2,3-dihydro-phenalen-l-olo-O-methyl ether;* **5** *4,5-dimethoxy -9-phenyl-2,3-dihydro-phenalen-1-ol-O-methyl ether;* **6**, *dimer of phenalene;* **7**, *dimer of phenalene;* **8**, *dimer of phenalene;* **9** *dimer of phenalene;* **10**, *dimer of phenalene;* **11**, *dimer of phenalene;* **12**, *2-hydroxy-8-(4-hydroxyphenyl)-phenalen-1-one;* **13**, *2-hydroxy-8-(3,4-dihydroxyphenyl)-phenalen-1-one;* **14**, *4,8,9-trimethoxy-1-phenyl-2,3-dihydro-1H-phenalene;* **15**, *4,8,9-trimethoxy-1-(4˝ methoxyphenyl)-2,3-dihydro-1H-phenalene;* **16**, *4,4˝,8,8˝,9,9˝-hexamethoxy-1,1˝-diphenyl-2,2˝,3,3˝-tetrahydro-7,7˝-bi(1H-phenalene);* **17**, *4,4˝,8,8˝,9,9˝,4´,4˝´-octamethoxy-1,1˝-diphenyl-2,2˝,3,3˝-tetrahydro-7,7˝-bi(1H-phenalene);* **18**, *6,6˝,8,8˝,9,9˝,4´,4˝´-octamethoxy-1,1˝ diphenyl-2,2˝,3,3˝-tetrahydro-7,7˝-bi(1H-phenalene);* **19**, *dimethyl 3-(4 -methoxyphenyl)naphthalene-1,8-dicarboxylate;* **20**, *methyl 5-methoxy-2-phenyl-8-(3,7,10-trimethoxy-6-phenyl-5,6-dihydro-4H-phenaleno[2,1-b]furan-9-yl)-1-naphthoate;* **21**, *2,3-dihydro-4,9- dihydroxy-8-methoxy-1-phenylphenalene;* **22**, *2,3-dihydro-3,9-dihydroxy-5-methoxy-4-phenyl-1H-phenalen-1-one;* **23**, *2,3-dihydro-8-methoxy-9-phenyl-1H-phenalene-1,4-diol;* **24**, *2,3-dihydro-4,8-dimethoxy-9-phenyl-1H-phenalen-1-ol;* **25**, *2,3-dihydro-9-(4-hydroxyphenyl)-8-methoxy-1H-phenalene-1,4-diol;* **26**, *2,6-dimethoxy-9-phenyl-1H-phenalen-1-one;* **27**, *7-(4-hydroxyphenyl)-5,6-dimethoxy-1H-phenalen-1-one;* **28**, *2-hydroxy-9-(4-hydroxyphenyl)-1H-phenalen-1-one;* **29**, *5,6-dimethoxy-7-phenyl-1H-phenalen-1-one;* **30**, *2-hydroxy-8-(4-hydroxyphenyl)-1H-phenalen-1-one;* **31**, *methyl 3-(4-hydroxy-3-methoxyphenyl)prop-2-enoate (methyl ferulate);* **32**, *4α-methyl-5α-ergosta-8,14,24(28)-triene-3β,4β-diol;* **33**, *4α-methyl-5α-ergosta-8,24(28)-diene-3β,4β-diol;* **34**, *4α-methyl-5α-ergosta-7,24(28)-diene-3β,4β-diol;* **35**, *6α-hydroxystigmata-4,22-dien-3-one;* **36**, *2-phenyl-5-methoxy-1,8-naphthalenedicarboxylic acid dimethyl ester;* **37**, *2-(p-methoxyphenyl)- 5-methoxy-1,8 naphthalenedicarboxylic acid dimethyl ester;* **38**, *2-phenyl-6-methoxy-1,8-naphthalenedicarboxylic acid dimethyl ester;* **39**, *2-phenyl-1,8-naphthalene dicarboxylic acid dimethyl ester linked at C-5 to a phenalenol;* **40**, *1,8-naphthalene dicarboxylic acid dimethyl ester linked at C-5 to a phenalenol.*

2.2.4.8 Other metabolites in *E. crassipes*

Resins (**Viado, 2006**), saponins in chloroform and methanol extracts (**Ndubuisi *et al,* 2007; Vasu *et al,* 2009**) and anthraquinones in the chloroform extract (**Lakshminarayana *et al,* 1984**) of *E. crassipes* are documented. The aqueous extract of *E. crassipes* shoot contains phlobatannin, quinone, anthraquinone and cardiac glycosides, whereas phlobatannin and cardiac glycosides are absent in the rhizome (**Lata and Dubey, 2010a**).

Cyclic voltammetric and titration methods show the presence of ascorbic acid in *E. crassipes*. By the cyclic voltammetric method the yield was 10.2%, and by the titrometric method, 16.3 mg/100 g (**Ogunlesi *et al,* 2010**). Ascorbic acid (AA), iso-ascorbic acid and dehydroascorbic acid was detected in the ethanol and light petroleum extracts of the shoot, whereas only dehydroascorbic acid was present in the rhizome. Quinones, such as 7-methyljuglone, rhein and aloe-emodin were found in the shoot, whereas aloe-emodin was present in the rhizome (**Lata and Dubey, 2010e**).

Pectin was prepared from *E. crassipes* in high yield by extracting dried *E. crassipes* with water, then ammonium oxalate and subsequently with HCl followed by NaOH (**Ishii *et al,* 1987**). The tissues of *E. crassipes* contain many air-filled intercellular spaces, which soak up water, but are also tough as a result of fibers, which contain sharp needles of calcium oxalate (**Bolenz *et al,* 1990**).

Surface waxes from the chloroform extract of *E. crassipes* leaves yield olefins. The alkanes separated from the waxes through column chromatography were identified by gas chromatography (**Amaral *et al,* 1990**). Five 1,8-naphthalenedicarboxylic acid dimethyl esters (List 1. **36-40**) were isolated from the acidic portion of the ethylacetate extract of *E. crassipes*. An oxidative process might transform phenylphenalenones into phenylnapthalic acid and a subsequent hydrolysis of these compounds might give phenyl-1,8-naphthalene dicarboxylic acid (**Greca *et al,* 1993**).

Alkaline treatment at high temperature, enzymatic hydrolysis and fermentation of *E. crassipes* yields 2,3-butanediol. Prefermentation hydrolysis followed by combined hydrolysis and fermentation has been found to be more efficient than combined hydrofermentation and separate hydrolysis fermentation, yielding 15 g of 2,3-butanediol per 100 g of plant

(**Motwani *et al,* 1993**). Roots of waterhyacinth extracted successively with solvents of increasing polarity *viz*. diethyl ether, acetone and ethyl acetate showed the best antialgal activity. β-D-dehydrated pyranose, pelargonic acid, isocyanoethyl acetate, 2,2-dimethyl cyclo pentanone, and propane amide isolated from the acetone extract by column chromatography showed inhibitory activity against algae (**Jin *et al,* 2003**). Leaves, stems and roots of the plant contains humic acids, which play an important role in water retention, metal and organic solute binding functions, and texture/workability in soils (**Ghabbour *et al,* 2004**). Acid-catalyzed hydrolysis of waterhyacinth yields levulinic acid at higher sulfuric acid concentrations, and propionic acid at lower concentrations (**Girisuta *et al,* 2008**).

E. crassipes oil contains both phenethyl alcohol and benzyl alcohol. These substances are found in many essential oils and have a variety of uses (**www.webnawab.com**).The presence of N1-acetyl-N2-formyl-5-methoxykynuramine and its precursor melatonin (**Tan *et al,* 2007**), which are potent free radical scavengers, assist plants in coping with harsh environmental insults (**Tan *et al,* 2007**). The S- and R-enantiomer of fipronil in *E. crassipes* was detected by HPLC. Fipronil-sulfone and fipronil-sulfide, the metabolites of fipronil, were detected by GC-MS to show the main metabolic pathway of fipronil (**Lu *et al,* 2010**). The acetone extract yielded *N*-phenyl-2-naphthylamine, linoleic acid and glycerol-1,9-12 (ZZ)-octadecadienoic ester. These compounds possess antialgal activities, with that of *N*-phenyl-2-naphthylamine being greater than that of the common algicide $CuSO_4$ (**Yang *et al,* 1992**).

2. 3 Biological and Pharmacological studies

2.3.1 Plants as antimicrobial agents

Infectious diseases represent an important cause of morbidity and mortality among the general population, particularly in developing countries. Plants are a source of variety of metabolites that are being used as drugs. Many plants have been studied for their activity in past few years indicating the surge of the researchers to identify a plant with good antimicrobial activity (**Cutler and Wilson, 2004; Rios and Recio, 2005; Prabuseenivasan *et al*, 2006; Leeja and Thoppil, 2007; Satish *et al*, 2007; Kanan and Al-Najar, 2008; Parekh and Chanda, 2008; Satish *et al*, 2008; Arunkumar and Muthuselvam, 2009; Babu and Subhasre, 2009; Bhardwaj and Laura, 2009; Meena *et al,* 2009; Ajayi and Akintola, 2010; Khan and Nasreen, 2010; Pavithra *et al*, 2010; Silva and Júnior,**

2010; Solanki, 2010; Kumar *et al*, 2011; Pelegrini *et al,* 2011; Tiwari *et al*, 2011; Labed *et al*, 2012; Vashist and Jindal, 2012; Vio-Michaelis *et al*, 2012).

2.3.1.1 Review related to the activity of plant extracts against *Staphylococcus albus*

Ethanolic extract of rhizome of *Curcuma longa* Linn (**Chauhan *et al,* 2003**), stem and leaves of *Peperomia galioides* (**Langfield *et al,* 2004**), solvent extracts of *Clerodendrum inerme* (**Khan and Khan, 2005**), butanol and ethanol extract of the leaves and bark of *Wrightia tomentosa* (**Nagarajan *et al,* 2006**), methanol extracts of *Cryptolepis buchanani*, *Mangifera indica*, *Manilkara hexandra* and *Nyctanthes arbor-tristis* extracts and few other plants (**Mahida and Mohan, 2007**), ethanolic leaf extract of *Ficus exasperate* (**Odunbaku *et al,* 2008**), extracts of *Solanum xanthocarpum* (**Salar and Suchitra, 2009**) were reported to be active against *Staphylococcus albus*.

Aqueous extract of *Moringa oleifera* leaf stalk (**Thilza *et al,* 2010**), methanol and aqueous extract of fifty-seven seaweed species from the coast of South Korea (**Choi *et al,* 2011**) and the aqueous leaf extract of *C.alata* (**Saito *et al,* 2012**) were reported to possess antibacterial against *Staphylococcus albus.*

2.3.1.2 Antibacterial activity of plant extracts against *Pseudomonas aeruginosa*

Methanol extracts of *Dianthus caryophyllus* and *Terminalia chebula* (Gaertner) (**Bonjar *et al,* 2003**), methanolic leaf extracts of *Eucalyptus camaldulensis* and *Terminalia catappa* (**Babayi *et al,* 2004**), root bark extracts of *Annona senegalensis, Securidacca longipendiculata* and *Steganotaenia araliacea* (**Lino and Deogracious, 2005**), water, ethanol and methanol extracts of single and combined plants like *Rhus coriaria* and *Thymus vulgaris* (**Adwan *et al,* 2006**), solvent extract of *Semicarpus anacardium L.f*. (**Mohanta *et al,* 2007**), aqueous extracts of *Conocarpus erectus*, *Callistemon viminalis* and *Bucida buceras* (**Adonizio *et al,* 2008**) were shown to be active against *Pseudomonas aeruginosa.*

Aqueous, chloroform and ethanolic extracts of leaf of *Senna siamea* (Lam) (**Bukar *et al,* 2009**), ethanolic extracts of amla (*Emblica officinalis*), neem (*Azadirachta indica*) leaves, aloe (*Aloe vera*) leaves, Assam tea (*Camellia sinensis assamica*) leaves and clove (*Syzygium aromaticum*) (**Mehrotra *et al,* 2010**), aqueous and methanolic extracts of pomegranate fruit skin (**Sadeghian *et al,* 2011**) and water and ethanol extracts of *Tribulus*

terrestris (**Deshwal, 2012**) are reported as antibacterial agents against *Pseudomonas aeruginosa*.

2.3.1.3 Antibacterial activity of *E. crassipes* against *Pseudomonas aeruginosa*

Chloroform, ethanol, methanol and aqueous extract of the roots and leaves of *E. crassipes* were found to show activity against *Pseudomonas aeruginosa* (**Fareed *et al,* 2008**).

2.3.1.4 Antifungal activity of plant extracts against *Aspergillus niger*

n-Hexane and methanol extract of *Eupatorium aschenbornianum* and *Sadium oxypetalum*, *Lysiloma acapulcensis* and *Annona cherimolia* (**Garcia *et al,* 2003**), extracts of *Lotus garcinii* (**Hiort *et al,* 2004**), hot water extracts of leaf and seed of Uda (*Xylopia aethiopica*) and Ginger (*Zinigiber officinale* (**Okigbo and Nmeka, 2005**), extracts from the leaf of *Solanum tomentosum* (**Aliero and Afolayan, 2006**), aqueous, ethyl alcohol and acetone extracts of *Allium sativum*, *Allium cepa* and *Allium porrum* (**Irkin and Korukluoglu, 2007**), oils of *Cuminum cyminum, Allium sativum, Ocimum sanctum, Trachyspermum copticum, Foeniculum vulgare* and *Elettaria cardamomum* (**Bansod and Rai, 2008**) were found to be active against *Aspergillus niger.*

Methanolic extracts of forty nine traditional Indian medicinal plants (**Bobbarala *et al,* 2009a**), aqueous extracts of different parts of *Syzygium aromaticum* and *Allium sativum* (**Avasthi *et al,* 2010**), aqueous, ethanolic and ethyl acetate leaf extract of neem (**Mahmoud *et al,* 2011**) and the methanolic leaf extracts of three plant species *Acacia modesta Wall* (Phulai), *Prosopis cineraria and Prosopis juliflora* (**Napar *et al,* 2012**) were found to show antifungal activity against *Aspergillus niger*.

2.3.1.5 Antifungal activity of *E. crassipes* against *Aspergillus niger*

Chloroform, ethanol, methanol and aqueous extract of the roots and leaves of *E. crassipes* (**Fareed *et al*, 2008**) showed significant activity against *Aspergillus niger*. Methanol extract of the plant also showed significant reduction in the growth of *Aspergillus niger* (**Bobborola *et al,* 2009a**). Methanol extract and its TLC fractions of *E. crassipes* did not inhibit the growth of *Aspergillus niger* (**Shanab *et al*, 2010; Shanab and Shalaby, 2012**).

2.3.1.6 Antifungal activity of plant extracts against *Mucor* sp.

Chloroform, petroleum ether, butanol and aqueous extracts of dried and powdered flowers, stems and leaves of *Euphorbia macroclada* (**Mughrabi *et al,* 2003**), 95% ethanol extract of six medicinal plants from (**Khalil *et al,* 2005**), petroleum ether, ethyl acetate, chloroform and methanol extract of root, stem and leaves of *Piper longum* (**Ali *et al,* 2007**), water, petroleum ether and ethanol extracts of three medicinal plants (**Ubalua and Oti, 2008**), ethanolic extracts of *Trikatu churna* and its individual components (**Reddy and Seetharam, 2009**), ethanol (95%), methanol (95%) and aqueous extracts of black pepper and turmeric (**Pundir and Jain, 2010**), essential oil of *Syzygium aromaticum* (**Rana *et al,* 2011**) and the ethyl acetate crude extracts of 28 selected endophytic fungi (**Powthong *et al,* 2012**) showed antifungal activity against *Mucor* sp. **There are no reports on the antifungal activity of *E. crassipes* against *Mucor* sp.**

2.3.1.7 Antimicrobial activity of extracts of *E. crassipes* against other microorganisms

Many researchers have evaluated the antimicrobial activity of various extracts of the *E. crassipes*. The pharmacognostical details of the root and rhizome viz. macro and microscopical studies, along with preliminary phytochemical tests and fluorescence analysis have been reported (**Sharma and Nayar, 1980**).

Significant antimicrobial activity is reported for various extracts of *E. crassipes* against more than fifty microorganisms like *Brenneria nitgrifluens, Colletotrichum graminicola, Serratia liquefaciens etc.,* (**Fareed *et al,* 2008**; **Bobbarala *et al,* 2009**; **Zhou *et al,* 2009**; **Baral *et al,* 2010**; **Shanab *et al,* 2010; Vadlapudi, 2010**; **Shanab and Shalaby, 2012**).

2.3.2 Antioxidant activity

Free radicals (pro-oxidants) in the form of reactive oxygen species and nitrogen species are produced in our body due to various biological processes as well as due to some exogenous factors like tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides (**Joseph *et al,* 2009**). These reactive species react with biomolecules, causing cellular injury and death (**Wong *et al,* 2006**).

Organisms contain a complex network of antioxidant molecules like glutathione, Vitamin C and vitamin E together with enzymes like catalase, superoxide dismutase and various peroxidases that work to prevent oxidative damage of cellular components like

DNA, proteins and lipids. Antioxidants work either by preventing the formation of the free radicals or by inhibiting them before they can damage the cellular components.

Naturally, there is a dynamic balance between the amount of free radicals generated in the body and antioxidants to quench and/or scavenge them and protect the body against their deleterious effects. However, the amount of these protective antioxidant principles present under the normal physiological conditions, are sufficient only to cope with the physiological rate of free radical generation. It is obvious, therefore, that any additional burden of free-radicals either from environment or produced within the body, can tip the free radical (pro-oxidant) and anti-free radical (antioxidant) balance leading to oxidative stress, which may result in tissue injury and subsequent diseases. Synthetic antioxidants like Butylated Hydroxy Anisole (BHA), Butylated Hydroxy Toluene (BHT) were extensively used to prevent the damage caused by these free radicals but due to the problems caused by these synthetic antioxidants, researchers started looking in to possible ways to identify a potent antioxidant (**Zakaria *et al,* 2011**). Plants have been reported to be the source of antioxidants because of the presence of phytochemicals including polyphenolics and flavonoids (**Agbafor and Nwachukwu, 2011**).

Antioxidant capacity of any substance can be evaluated both *in vivo* and *in vitro*. The *in vitro* models for evaluating the antioxidant activity include Conjugated diene assay, DPPH assay (1, 1 diphenyl 2, picryl hydrazyl), Super oxide radical scavenging activity, Hydroxyl radical scavenging activity, Nitric oxide radical inhibition activity, Reducing Power Method, Phospho molybdenum Method, Peroxynitrite radical scavenging activity, ABTS (2, 2-azinobis (3-ethyl benzothiazoline- 6- sulfonic acid) diamoniumsalt) Method, DMPD (N, N-dimethyl-p-phenylene diamine dihydrochloride) Method, Oxygen Radical Absorbance Capacity (ORAC), β-Carotene Linoleate model, Xanthine oxidase Method, FRAP (Ferric Reducing Ability of Plasma) Method, TRAP (total radical trapping antioxidant parameter) Method, Cytochrome C test, Erythrocyte ghost system, Microsomal lipid peroxidation or Thiobarbituric acid (TBA) assay (**Joseph *et al,* 2010**). Among these, the most widely used assays include DPPH scavenging assay and reducing power assay as these are convenient and feasible methods (**Chanda and Dave, 2009**).

2.3.2.1 DPPH radical scavenging assay

α, α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging method offers the first approach for evaluating the antioxidant potential of a compound, an extract or other biological sources. The assay is based on the measurement of the scavenging capacity of antioxidants towards it. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine. The delocalisation of the spare electron takes place over the molecule as a whole so that the molecules do not dimerise, like most other free radicals. The delocalisation also gives rise to the deep violet colour, with an absorption in ethanol solution at around 520 nm. On mixing DPPH solution with a substance that donates a hydrogen atom, it gives rise to the reduced form with the loss of violet colour. While DPPH accepts an electron or hydrogen radical to become a stable, diamagnetic molecule, it can be oxidized only with difficulty, and then irreversibly (**Pattanayak *et al,* 2012**). DPPH shows a strong absorption band at 517 nm due to its odd electron and solution appears a deep violet colour. The absorption vanishes as the electron pairs off (**Awika *et al,* 2003; Lu *et al,* 2003; Jayashree *et al,* 2008**) resulting in yellow colour (**Wang *et al,* 2011**). The resulting decolorization is stoichiometric with respect to the number of electrons taken up. The alcoholic solutions of 0.5 mM are densely coloured and at this concentration, the Lambert-Beer law is obeyed over the useful range of absorption (**Kedare and Singh, 2011**).

2.3.2.2 Reducing power assay

The reducing capacity of a compound serves as a significant indicator of its potential antioxidant activity (**Oktay *et al,* 2003**). Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (**Yen and Chen, 1995**). This method is based on the principle of increase in the absorbance of the reaction mixtures. Increase in the absorbance indicates increase in the antioxidant activity. In this method antioxidant compound forms a colored complex with potassium ferricyanide, trichloro acetic acid and ferric chloride, which is measured at 700 nm. Increase in absorbance of the reaction mixture indicates the reducing power of the samples (**Joseph *et al,* 2010**).

2.3.2.3 DPPH$^{\cdot}$ radical scavenging activity and Reducing power assay of plant extracts

The DPPH$^{\cdot}$ scavenging ability of various extracts of propolis, (**Lu *et al,* 2003**), *Calocedrus formosona Florin* (**Wang *et al,* 2004**), *Elaeis guineensis* (**Balasundram *et al,* 2005**), *Chamaecyparis lawsonia* (**Gao *et al,* 2006**), *Carica papaya*, *Magnifera indica*, *Psidium guajava* and *Vernonia amygdalina* (**Ayoola *et al,* 2008**), *Amphimas pterocaroides, Harungana madagascariensis, Myrianthus arboreus,* and *Cussonia barteri* (**Biapa *et al,* 2007**) is documented. Extracts of *Ocimum gratissimum* (**Akinmoladun *et al,* 2007**), *Fumaria capreolata* and *Fumaria bastardii* (**Benabdesselam *et al,* 2007**) have been reported to possess DPPH$^{\cdot}$ scavenging ability and reducing capacity. *Elaeocarpus ganitrus* has been demonstrated to exhibit reducing capacity (**Sathishkumar *et al,* 2008**).

The DPPH$^{\cdot}$ scavenging assay and reducing power of extracts of *Portulaca oleracea* (**Sanja *et al,* 2009**) and *Costus pictus* (**Jayasri *et al,* 2009**) are reported. DPPH radical scavenging activity of *Evolvulus nummularius* (**Pavithra *et al,* 2009**), *Pandanus odoratissimus* (**Sasikumar *et al,* 2009**), *Drynaria quercifolia* (**Beknal *et al,* 2010**), *Capparis* (**Sini *et al,* 2010**) are documented. *Calotropis gigantea* (**Amit *et al,* 2010**), *Madhuca longifolia* (**Prashanth *et al,* 2010**), *Nyctanthes arbor-tristis* (**Thangavelu and Thomas, 2010**) has been demonstrated to exhibit DPPH radical scavenging ability and reducing ability.

Hyssopus officinalis, *Colchicum speciosum*, *Viola odorata* and *Buxus hyrcana* (**Ebrahimzadeh *et al,* 2010**) were demonstrated to exhibit DPPH$^{\cdot}$ scavenging ability and reducing power ability. DPPH$^{\cdot}$ scavenging activity of extracts of *Curcuma zedoaria* (**Himaja *et al,* 2010**), *Lespedeza cuneata* (**Kim and Kim, 2010**), *Kaempferia galanga* (**Sumazian *et al,* 2010**), *Vitex doniana* and *Mucuna pruriens* (**Agbafor and Nwachukwu, 2011**) is reported.

Extracts of *Acacia nilotica* (**Gowri *et al,* 2011**), *P.emblica* (**Jamuna *et al,* 2011**), *Garcinia pedunculata* (**Devi *et al,* 2012**) *Zizyphus oenoplia* (**Jadav and Chavan, 2012**) and *Achyranthes aspera* (**Kumar *et al,* 2012**) exhibited a dose dependent increase in the DPPH$^{\cdot}$ scavenging ability and reducing power. *Clitoria ternatea*, *Solanum nigrum* and *Aloe vera* exhibited DPPH radical scavenging ability (**Jayachitra and Krithiga, 2012**).

2.3.2.4 Literature pertaining to the antioxidant activity of *E. crassipes*

E. crassipes displays effective antioxidant activity and the glutathione content of the plant is 32 ± 1.6 nmol g^{-1} of dry hyacinth leaves, which corresponds to ca. 3.2 nmol per

gram of fresh leaves (**Bodo *et al,* 2004b**). The ethanol extract contains higher amounts of polyphenols [6.68 mg gallic acid equivalents (GAE)/g] in the leaf than in the stem (4.37 mg GAE/g). Waterhyacinth also contains higher contents of flavonoids [1524 and 453 mg rutin equivalents g^{-1} of leaf and stem, respectively]. Accordingly, both the leaf and stem have high reducing power [118.3 and 47.2 10 µg ascorbic acid equivalents g^{-1}, respectively] and DPPH radical scavenging capability (74.6% for leaf and 62.7% for stem) (**Liu *et al,* 2010**).

E. crassipes exposed to various concentrations of Ag, Cd, Cr, Cu, Hg, Ni, Pb and Zn hydroponically for 21 days showed increase in the activity of catalase, peroxidase and superoxide dismutase, and there was differential inducement among the metals. Overall, Zn had the least inducement of the antioxidant enzymes in *E. crassipes* and *Pistia stratiotes*, while Hg had the highest inducement (**Odjegba and Fasidi, 2007**).

Ethanol extract of *E. crassipes* at a dose of 50% was observed to be most effective in the treated rats and reduced the arsenic accumulation in liver, spleen, kidney, intestine, lungs and skin. Besides, it reduced the oxidative stress caused by arsenic as is evident by decreased levels of malondialdehyde (MDA) in the above organs (**Quayum, 2007**).

The antioxidant activity of *n*-hexane, ethyl acetate and methanol extracts by $DPPH^{\cdot}$ and ABTS assays revealed that the activity is concentration dependent. In the DPPH assay, the ethyl acetate extract showed greater activity than the methanol and *n*-hexane extracts, whereas in the ABTS assay the methanol extract recorded the higher activity. The crude methanolic extract showed higher activity than its fractions (**Shanab *et al,* 2007**). The antioxidant activity of *E. crassipes* by the $DPPH^{\cdot}$ scavenging method showed appreciable activities and this was attributed to the presence of hydroxyl groups and unsaturated bonds in the structure of the compounds (**Enien *et al,* 2011**).

The ethanol and aqueous extract of *E. crassipes* evaluated by DPPH radical scavenging ability, iron chelating activity, reducing power and prevention of oxidation in a liposome model system demonstrated the efficiency of ethanol extract compared to aqueous extract. The IC_{50} for the water and methanol extract of *E. crassipes* in DPPH radical scavenging activity was greater than 2,000 mg for both the extracts (**Ho *et al,* 2012**). The reducing power of the aqueous and methanol extract of *E. crassipes* was 0.17

± 0.01 and 0.26 ± 0.01 (*Δ* 700). Trolox-equivalent antioxidant capacity of aqueous and methanol extract of *E. crassipes* was 102.13 ± 2.66 and 143.33 ± 6.39 (μM Trolox/mg ± SD) respectively (**Ho *et al,* 2012**).

Antioxidant activity depends on the phytochemicals which also depend on the method of extraction. There are no reports on the solvent extracts obtained by different extraction methods.

2.3.3. Acute toxicity studies

Any drug before being used by human must be proved to be non-toxic. There are many ways by which the toxicity of the drugs can be determined. Acute toxicity is that produced after administration of a single dose (or multiple doses) in a period not exceeding 24 h, up to a limit of 2000 mg/kg. The main objective of acute toxicity test is to identify a dose causing major adverse effects and an estimation of the minimum dose causing lethality, according to regulatory guidelines (**Chapman, 2007**). Data from the acute study may: (a) Serve as the basis for classification and labelling; (b) Provide initial information on the mode of toxic action of a substance; (c) Help arrive at a dose of a new compound; (d) Help in dose determination in animal studies and (e) Help determine LD_{50} values that provide many indices of potential types of drug activity (**Akhila *et al,* 2007**).

2.3.3.1 Review related to acute oral toxicity studies of plant extracts

Acute oral toxicity studies (LD_{50}) of crude aqueous ethanolic extract of the roots of *Hemidesmus indi var. pubescens R. Br.* was evaluated in Swiss Albino mice of either sex (**Austin and Jegadeesa, 2002**). The LD_{50} of the methanolic extract of the root-bark of *Fagara zanthoxyloides* was found to be in mice at 5.0 g/kg body weight within 95 % confidence limits (**Ogwal-Okeng *et al,* 2003**).

Acute toxicity study of the ethanolic rhizome extract of *Kaempferia galangal* was studied by oral administration of 5.0 g/kg of the extract to the groups of adult Sprague-Dawley rats of either sex, in a single oral dose which produced neither mortality nor significant differences in the body and organ weights between controls and treated animals, indicating the non toxic nature of the extract (**Kanjanapothi *et al,* 2004**). Toxicity of *Wedelia paludosa* evaluated in Swiss mice after ingestions of the extract during one day (acute model), indicated the LD_{50} to be higher than 4000 mg/kg (**Burger *et al,* 2005**). The acute toxicities of hydro-ethanolic extract of leaves of *Senna alata* in Swiss mice showed

that the medium lethal dose (LD_{50}) was about 18.50 g/kg of body weight (**Pieme *et al,* 2006**).

Aqueous extract of dried fruits of *Piper nigrum* assessed for its acute toxicity in male and female Sprague-Dawley rats showed it to be non-toxic upto 5,000 mg/kg body weight after an acute exposure (**Chunlaratthanaphorn *et al,* 2007**). Acute toxicological studies of the aqueous extract of Iraqi *Peganum harmala* in Albino Wistar rats indicated that the LD_{50} value of the extract in rats given intramuscularly was 420 mg/kg. Tremor and convulsion were observed in most of the treated rats specially those given large dose due to the alkaloid content of the extracts which have a central nervous system stimulant effects (**Muhi-eldeen *et al,* 2008**).

Oral LD_{50} of the aqueous seed extract of *Persea Americana* was indeterminable being in excess of 10 g/kg in adult male rats (**Ozolua *et al,* 2009**). The median oral acute toxicity value (LD_{50}) of *C.ordorata* leaves extract in Swiss Albino mice was determined to be 16.50 g/kg body weight (**Ogbonnia *et al,* 2010**).

The aqueous extract from the fruits of *Piper chaba* evaluated for its acute toxicity in both male and female rats in a single oral dose of 5,000 mg/kg body weight showed the extracts to be non toxic (**Jaijoy *et al,* 2011**). LD_{50} of the ethanol extract of the *Z. zerumbet* rhizomes *via* the oral route in Wistar rats of both sexes tested was found to be 15 g kg^{-1} (**Chang *et al,* 2012**).

The methanolic extract (50%) of *E. crassipes* tested in hybrid male mice (from *Swiss* albino female and C57BL male) showed the LD_{50} to be greater than 500 mg/kg as there was no mortaility up to this dose (**Ali *et al,* 2009**). There are no reports on the acute oral toxicity of other solvent extracts of *E. crassipes*.

2.3.4 Wound healing activity

Wounds are the result of injuries to the skin that disrupt the other soft tissue. Healing of a wound is a complex and protracted process of tissue repair and remodeling in response to injury (**Diegelmann and Evans, 2004**). A number of factors like oxygenation, infection, age, stress and hormones affect wound healing (**Guo and DiPietro, 2009**). Various plant products have been used in treatment of wounds over the years. Wound healing herbal extracts promote blood clotting, fight infection, and accelerate the healing of wounds. Phytoconstituents derived from plants need to be identified and screened for antimicrobial

activity for management of wounds. Small animals provide a multitude of model choices for various human wound conditions (**Thakur *et al,* 2011**). *In vivo* models for evaluating the wound healing efficacy can be done by excision wound model, incision wound model, dead space wound model and burn wound model (**Kiran and Asad, 2008; Ananth *et al,* 2010**).

2.3.4.1 Plants as wound healing agents

Plants have long been used as wound healing agents and number of reviews have been published on the use of plants as wound healing agents (**Biswas and Mukherjee, 2003; Kumar *et al,* 2007; Ayyanar and Ignacimuthu, 2009; Sravanthi *et al,* 2010; Venkatanarayana *et al,* 2010; Pawar and Toppo, 2012; Reddy *et al,* 2012; Soni and Singhai, 2012**).

2.3.4.1.1 Review pertaining to *in vivo* wound healing study of plants

The ethanol extract of the shade-dried leaves of *Aristolochia bracteolata Lam.* studied for its effect on wound healing in Albino rats, using incision, excision and dead-space wound models, at two different dose levels of 400 and 800 mg/kg/body wt./day showed an enhanced wound healing activity due to the free radical scavenging activity of the plants and the enhanced level of antioxidant enzymes in the tissues (**Shirwaikar *et al,* 2003**). The methanol leaf extract of *Flabellaria paniculala* showed potential wound healing activity on normal and infected wounds in Wistar rats by excision model activity (**Abo *et al,* 2004**).

An alcoholic bark extract of *Butea monosperma* showed faster healing in male Albino Wistar rats on excision wounds. The extract treated wounds showed improved rate of epithelialisation, wound contraction and was confirmed by histopathological examinations (**Sumithra *et al,* 2005**). The flower extract of *Catharanthus roseus* showed an increased tensile strength and faster wound contraction in healthy inbred gender-matched Sprague Dawley rats (**Nayak *et al,* 2006**). The aqueous extract of *Carica papaya* fruit evaluated for its wound healing activity in streptozotocin-induced diabetic rats using excision and dead space wound models, exhibited a faster rate of epithelialisation, reduction in the wound area, increased hydroxyproline content and increased wet and dry granulation tissue weight when compared to controls (**Nayak *et al,* 2007**).

Effect of aqueous extract of leaves of *Morinda citrifolia* (200 mg/kg) on excision, resutured incision and dead space wound models showed a significant increase in wound

contraction rate, tensile strength, granuloma breaking strength, collagen content, dry granuloma weight (p<0.01) and hydroxyproline content. *M. citrifolia* was demonstrated to accelerate healing process by reducing lipid peroxide levels in treated wounds (**Rasal *et al,* 2008**). The wound healing property of the crude methanolic extract of *Cassia occidentalis* evaluated by incision model in male Albino rats showed a significant increase in skin breaking strength which was comparable with soframycin treated group (**Sheeba *et al,* 2009**).

Increased wound breaking strength, decreased epithelization period, increased wound contraction, granulation tissue weight and hydroxyproline concentration were noted in the Albino rats treated with the methanol extract of *Limonia acidissima* Linn fruit bulb (**Ilango and Chitra, 2010**). The methanolic extract of the peels and peel bandage of *Ipomoea batatas* tubers (sweet potato) was screened for wound healing by excision and incision wound models on Wistar rats. In the incision wound model, high tensile strength of the wounded skin was observed due to an increase in collagen concentration and stabilization of the fibers (**Panda *et al,* 2011**).

The methanolic extract of *E. crassipes* leaves in the form of an ointment, at two different concentrations (10% and 15%, w/w of leaf extract in a simple ointment base) was investigated for its wound healing potential in an excision model of wounds in male Wistar rats. The treatments showed better wound contraction ability that was significantly greater than that of the control (**Ali *et al,* 2010**). There are no reports on incision wound healing activity of the extracts of *E. crassipes*.

2.3.4.2 Review pertaining to wound healing by polyherbal formulations

The emulsifying ointment and carbopol 934 gel formulations containing extracts of *Azadirachta indica*, *Tridax procumbens* and *Curcuma longa* showed faster wound healing activity on experimentally induced excision wounds in Albino rats (**Bhat *et al,* 2007**).

A poly-herbal formulation containing aqueous lyophilized leaf extracts of *Hippophae rhamnoides* and *Aloe vera* and the ethanol rhizome extract of *Curcuma longa* in an optimized ratio (1:7:1) was studied in normal and impaired diabetic rats using a full-thickness cutaneous wound model. The formulation increased the cellular proliferation and collagen synthesis at the wound site in normal rats and hydroxyproline and hexosamine

content in the streptozotocin-induced diabetic rats together with wound contraction (**Gupta *et al,* 2008**).

Polyherbal formulation containing roots of *Ageratum conyzoides*, stem-bark of *Ficus religiosa*, rhizomes of *Curcuma longa* and leaves of *Tamarindus indica*, on excision wound model in Albino rats showed better wound healing than *Ageratum* treated groups. The synergistic action of Ageratum constituent and the constituent of other plants present in the polyherbal formulation contributed to enhanced wound healing (**Sachin *et al,* 2009**).

An indigenous herbal formulation containing *Comphora officinarum* (Kafoor), *Shorea robusta* (Raal), Beeswax (*Apis mellifera*), *Acacia catechu*, *Sesamum indicum* and *Azadirachta indica* (Neem oil) in excision and incision wound models using Albino rats showed a significantly higher contraction rate, shortened epithelisation period and significant increase in tensile strength ($p < 0.001$) (**Shafiuddin *et al,* 2009**). The ointment formulations containing extracts of *Curcuma longa, Tridax procumbens* and *Eclipta alba* on the experimentally induced open wound in Albino rats showed a faster rate of wound contraction than the control (**Srivastava and Mishra, 2009**).

Aloe vera, Aristolochia bracteata and *Curcuma longa* based traditional polyherbal formulation tested on surgically excised wounds in Wistar rats demonstrated a significant reduction in wound area as compared to the control animals (**Peer *et al,* 2010**). The wound healing activity of a gel containing cow ghee and *Aloe vera* was evaluated for its wound healing activity in rats by incision and excision wound models showed that the topical application of the test formulation promoted the tensile strength (incision wounds) and wound contraction (excision wounds) and the healing potential was comparable to framycetin sulphate cream (1%w/w). Histological examination revealed good keratinization, epithelization, fibrosis and collagenation indicative of the wound healing potential of gel (**Nandanwar *et al,* 2010**).

Six polyherbal ointments containing methanol leaf extracts of *Ageratum conyzoides*, *Argemone mexicana*, *Heliotropium indicum* and bark extract of *Alstonia scholaris* tested for wound healing activity in rats using excision and incision wound models and skin irritation study demonstrated an increased percentage of wound contraction, higher hydroxyproline

content and skin breaking strength compared to the Nitrofurazone (standard) (**Dash and Murthy, 2011**).

2.3.5 Skin whitening assay and Anti-ageing assay

The skin is the largest organ in our body and it plays an extremely important role, providing a vast physical barrier against mechanical, chemical, thermal and microbial factors that may affect the physiological status of the body. Over exposure of skin to sun light and pollution causes the production of reactive oxygen species which can react with DNA, proteins and fatty acids, causing an oxidative damage and impairment of antioxidant system. The formation of ROS in skin leads to various skin diseases predominantly wrinkles, ageing *etc.,* Cosmetic products are used for treating such conditions thereby improving the skin condition (**Roy *et al,* 2012**). Before being used as cosmeceutics, the cream has to be tested for various properties like stability, pH, total fat *etc.,* Many plant extracts are used in the form of cream in cosmeceutical industry and are evaluated by various assays.

2.3.5.1 Literature related to skin cream formulation with plant extracts

Antityrosinase activity of *Artocarpus lakoocha*, *Glycyrrhiza glabra* and *Bombyx mori* extract and the lotion prepared with these extracts were tested for its stability, tyrosinase inhibitory activity, TLC, colour and odour of the formulation at different temperatures for 4 week. The percentage of tyrosinase inhibition was highest for *Artocarpus lakoocha* followed by *Glycyrrhiza glabra* and *Bombyx mori* with no change in the activity of the cream upto four weeks of storage at 4 $^{\circ}$C (**Pothitirat *et al,* 2010**).

The tyrosinase inhibitory effect of formulations containing synthetic ellagic acid, *Juglans regia* leaves, *Castanea sativa* stem bark, and *Eucalyptus camaldulensis* plant extracts, and a combination of these showed gel formulation to be effective carrier for treating uneven skin pigmentation (**Ozer *et al,* 2007**). The skin cream and vehicle prepared with the hydroalcoholic extract of *Calendula* plant applied tested on human volunteers indicated the aesthetic nature of the skin creams and the positive rejuvenating effect on human skin (**Akhtar *et al,* 2011**).

Different types of formulation oils with the extracts of natural products such as *Aloe vera*, *Cucumis sativus* and *Daucus carota* were formulated by incorporating different

concentrations of stearic acid and cetyl alcohol. Formulations containing lesser quantity of stearic acid and higher quantity of cetyl alcohol showed phase separation and ease of removal, spreadibility, no inflammation and irritation, no redness and oedema (**Aswal *et al,* 2012**).

Cream containing 4% mulberry fruit ethanol extract (*Morus alba*) with base as control decreased the skin melanin content and skin erythema (**Akhtar *et al,* 2012**). Ethanolic extract (70%) of *Pleurotus ostreatus* was tested at different concentration for its $DPPH^{\cdot}$ scavenging and tyrosinase inhibition effect as gel and gel base showed increase in activity with increase in concentration of the gel. The $DPPH^{\cdot}$ scavenging effect of the gel was more than the extract whereas the tyrosinase inhibition ability was less (**Hapsari *et al,* 2012**).

2.3.5.2 Tyrosinase inhibition activity of plant extracts

Aqueous ethanolic extract (70%) of *Ramalus mori* evaluated for its tyrosinase inhibition effect in mushroom tyrosinase at various concentrations, showed high tyrosinase inhibition activity (**Lee *et al,* 2003**). Thalifoline, (-)-cubebin, ent-kauran-16b,17-diol, ent-16b,17-epoxykauran, ent-kaur-15-en- 17-ol isolated from the methanolic extract of fresh root and stem *Aristolochia elegans* Mast. were tested for its antityrosinase activity among whcih ent-kaur-15-en- 17-ol showed highest tyrosinase inhibition activity (**Shi *et al,* 2004**).

Ellagic acid enriched aqueous ethanol (50%) extract of Pomegranate fruit rind tested for its antityrosinase activity by DOPA chrome method exhibited an IC_{50} of 182.2 µg/mL (**Yoshimura *et al,* 2005**). Aqueous ethanolic extract (90%) of nine herbs tested for its tyrosinase activity in melan-a cells after treatment with 20 mg/mL herb extract was measured by L-dihydroxyphenylalanine (L-DOPA) staining of electrophoresed gel and *Radix Clematidis* was the most potent tyrosinase inhibitor among the nine herbs tested (**Zhong *et al,* 2006**).

The tyrosinase activity of the extracts of 53 parts of 36 plant species cultivated for edible and medicinal uses in Okinawa investigated for its antityrosinase activity indicated the potent activity of the extract of *Nandina domestica* (**Masuda *et al,* 2007**). Methanol extract of leaves of five *Etlingera* species analysed for tyrosinase inhibition activity using the modified dopachrome method with L-DOPA showed *E. elatior* as a potent inhibitor than the leaves of *Hibiscus tiliaceus,* positive control (43.9%) (**Chan *et al,* 2008**).

An 1:1 complex between polyvinylpyrolidone and the ethyl acetate extract of mangosteen pericarp inhibited the tyrosinase enzyme appreciably (**Tadtong *et al,* 2009**). Ethanolic extract (80%) of 299 parts of 263 plant species screened for tyrosinase inhibition activity using a 96-well reader demonstrated the effective activity of *Sophora flavescens* than other plants and positive controls (**Moon *et al,* 2010**). Ethanolic extract (70%) of *D.racemosum* extract showed highest tyrosinase inhibition than *Acer ginnala*, *Cornus walteri*, *Castanopsis cuspidate* var. *Thunbergi*i, *Acer psedo-siebolianum*, *Thuijopsis dolabrata*, *Cercidiphyllum Japonica* at different concentrations (**Kim *et al,* 2010**).

Aqueous, ethanol and petroleum ether extract of *Asparagus racemosa* tested by mushroom tyrosinase model for its antityrosinase activity showed that the aqueous extract of *Asparagus racemosa* (43.29%) exhibited maximum inhibiting potential of the tyrosinase enzyme (**Narayanaswamy *et al,* 2011**). Aqueous, methanol and petroleum ether extract of the bark, fruit, flower and leaves of *Mimusops elengi* were studied for their skin whitening potential through mushroom tyrosinase inhibition assay. The methanolic extract of *M.elengi* flowers showed the highest inhibition of tyrosinase with an IC_{50} value of 401 followed by methanolic extract of leaves (**Narayanasanamy *et al,* 2011**).

The aqueous extract of *Epilobium angustifolium* L. exhibited tyrosinase inhibitory activity that correlated with phenolic compound content (**Onar *et al,* 2012**). Methanol extract of *Musa sapientum* pericarp and spinage leaves (*Spinacia oleracea* folium) evaluated for its tyrosinase inhibition with L-tyrosine substrate, showed the extracts to be potent tyrosinase inhibitors due to the presence of polyphenols, such as flavonoids (**Heravi *et al,* 2012**).

2.3.5.3 Literature pertaining to DNA inhibition and DPPH$^{\cdot}$ radical scavenging assay of plant extracts

Camellia sinensis catechins showed Fe^{3+}/ascorbic acid/H_2O_2 induced 2-deoxyribose degradation while at higher concentrations (50 µM and 100 µM) there was an increase in MDA formation (15-30%) (**Raza and John, 2007**). Extracts of molasses studied for its antioxidant capacity and DNA oxidative damage by electrophoresis brought about protection against induced DNA oxidative damage (**Guimaraes *et al,* 2007**).

The IC_{50} of methanol extract of *Cyclotrichium niveum* aerial parts for DPPH$^{\cdot}$ scavenging activity and DNA damage protecting activity is 78.15 µg/mL (**Emen *et al,* 2009**). The aqueous extract among the methanolic, hexane and chloroform crude extracts of the whole plant of *Cyanthillium cinereum* showed highest DPPH$^{\cdot}$ scavenging assay and DNA

damage inhibition effect (**Guha *et al,* 2009**). *Cleome icosandra*, *Rosa damascena* and *Cyperus scariosus* showed significant oxidative DNA damage preventive activity and antioxidant activity (**Kalim *et al,* 2010**). Aqueous extract showed maximum radical scavenging in DPPH method among the hexane, chloroform, ethyl acetate, acetone and methanol extracts of Chicory (*Cichorium intybus*) leaf (**Ilaiyaraja and Khanum, 2010**).

The aqueous extract of *Asplenium ceterach* showed good DPPH radical scavenging activity of about 85% and pBR322 plasmid DNA damage inhibition in the presence of 20 mg/mL (**Berk *et al,* 2011**). Antioxidant properties of petroleum ether, hexane, ethyl acetate, chloroform, methanol and aqueous extracts of 10 selected *Asteraceae* species assessed by DPPH radical assay and pTZ57R/T plasmid DNA protection showed that the seven plants had highest antioxidant activity (**Chethan *et al,* 2012**).

The DPPH radical scavenging property and DNA damage protection by photolyzing pBR322 plasmid DNA for the hexane, chloroform, methanol and aqueous extracts from 14 medicinal plants had shown that the polar extracts had higher DPPH radical scavenging activity compared to the non-polar extracts (**Guha *et al,* 2011**). *Morinda tinctoria* has been reported to exhibit $DPPH^{\cdot}$ scavenging method (**Sreena *et al,* 2011**). Ethanol (70%) extract of *Trigonella foenum* graecum investigated by $DPPH^{\cdot}$ scavenging assay and Bleomycin dependent DNA damage assay indicated the non-pro-oxidant activity of the plant (**Subhashini *et al,* 2011**).

Aqueous extracts of *Lantana camara* leaves exhibited high activity in DPPH radical scavenging assay and complete protection of pBR322 plasmid DNA during DNA damage inhibition assay (**Kalitha *et al,* 2012**).

2.3.6 Other pharmacological and biological activites of *E. crassipes*

A methanolic leaf extract of *E. crassipes* (50%) showed good response against B16F10 *in vivo* melanoma tumor bearing hybrid mice models (**Ali *et al,* 2009**). Methanol extract and its fractions demonstrated anticancer activity against liver, breast and cervix cancer cell lines (**Enien *et al,* 2011**). The anticoagulant activity of sulphated polysaccharide-rich extracts of *E. crassipes* showed considerable anticoagulant activity (**Santos *et al,* 2012**).

2.3.7 Larvicidal and pupicidal activity

Mosquitoes can transmit more diseases than any other group of arthropods and affect million of people throughout the world. WHO has declared the mosquitoes as “public enemy number one”. Mosquitoes cause many deadly diseases like malaria, dengue, filariasis etc., To prevent proliferation of mosquito borne diseases and to improve quality of environment and public health, mosquito control is essential. Synthetic insecticides have been widely used in the past for the control of this vector (**Ghosh *et al,* 2012**). But due the resistance of these target vectors against the synthetic insecticides and the effect of the insecticides on the non-target organisms and several other factors, there has been resurgence in the research of plant derived insecticides. Mosquitoes in the larval stage are attractive targets for pesticides because they breed in water and, thus, are easy to deal with them in this habitat. Many of the natural compounds have been found to possess an array of properties, including insecticidal activity, repellency, feeding deterrence, reproduction retardation and insect growth regulation against various mosquito species (**Kishore *et al,* 2011**).

2.3.7.1 Larvicidal and pupicidal activity of plant extracts

Larvicidal activity of extracts of 22 Australian and 12 Mexican plants (**Kim *et al,* 2002**), *Argemone mexicana* (**Sakthivadivel and Thilagavathy, 2003**), *Pleioblastus juxianensis, Bmchystachyum albostriatum, Phyllostachys platyglossa* and *Pleioblastus amarus among seven bamboo species* (**Cao *et al,* 2004**) *Rhinacanthus nasutus*, *Derris elliptica*, *Trigonostemon reidioides*, *Homalomena aromatica*, *Stemona tuberosa* and *Acorus calamus* (**Komalamisra *et al,* 2005**) is reported.

Larvicidal activity of extracts of *M. azedarach* (**Maciel *et al,* 2006**), A.*barbadensis* (**Maurya *et al,* 2007**), *Cryptomeria japonica* (**Cheng *et al,* 2008**), *Myracrodruon urundeuva* (**Sá *et al,* 2009**), *Citrus hystrix*, *Citrus reticulata*, *Zingiber zerumbet*, *Kaempferia galanga*, and *Syzygium aromaticum* (**Sutthanont *et al,* 2010**) *Ricinus communis* (**Babarinde *et al,* 2011**), *Abutilon indicum*, *Cleistanthus collinus*, *Leucas aspera*, *Murraya koenigii*, *Hyptis suaveolens Citrullus colocynthis* and *Sphaeranthus indicus* (**Arivoli *et al,* 2012**) against disease causing mosquitoes is demonstrated.

2.3.7.2 Literature related to efficacy of plant extracts against *C. quinquefasciatus*

The methanol, chloroform and ether extracts of *Euphorbia tirucalli* latex and stem bark were evaluated for larvicidal activity against laboratory-reared larvae of

C.quinquefasciatus (**Yadav *et al,* 2002**). Larvicidal activity of the oil-resin of *Copaifera reticulata* on *C.quinquefasciatus* demonstrated larvicidal activity against all the *C. quinquefasciatus* instars (**Silva *et al,* 2003**). Larvicidal efficacies of extracts of *Momordica charantia*, *Trichosanthes anguina*, *Luffa acutangula*, *Benincasa cerifera* and *Citrullus vulgaris* against the late third larval age group of *C.quinquefasciatus* have been documented (**Prabakar and Jebanesan, 2004**).

Ethanolic extract of *Centella asiatica* leaves evaluated for the larvicidal and adult emergence inhibition activity against mosquito *C.quinquefasciatus* under five constant temperatures in the laboratory showed an increase in toxicity with increase in temperature (**Rajkumar and Jebanesan, 2001**). The mosquito larvicidal activity of prepared tablets containing 5% and 10% *R. nasutus* methanol extract showed an LC_{50} values of 18.7 and 17.3 respectively (**Rongsriyam *et al,* 2006**).

Ageratina adenophora acetone extract tested for its larvicidal activity against *C. quinquefasciatus*. The 24 h LC_{50} value of the leaf extract of *A. adenophora* was found 227.20 ppm for *C. quinquefasciatus* (**Raj Mohan and Ramaswamy, 2007**). Methanol leaf extracts of *V. trifolia* showed highest larvicidal activity among *Vitex negundo*, *Vitex peduncularis* and *Vitex altissima* (**Kannathasan *et al,* 2007**).

Moderate larvicidal effects were noted for the crude hexane, ethyl acetate, petroleum ether, acetone and methanol extracts of *Abutilon indicum*, *Aegle marmelos*, *Euphorbia thymifolia*, *Jatropha gossypifolia* and *Solanum torvum* against the early fourth-instar larvae of *C.quinquefasciatus*. β-sitosterol isolated from *A.indicum* exhibited potential larvicidal activity (LC_{50}-26.67 ppm) against *C. quinquefasciatus* Say (**Rahuman *et al,* 2008**). *C.papaya* seed extract followed by *M. paniculata* fruit extract, M*. paniculata* and *C. collinus* leaf extract showed larvicidal activity against *C. quinquefasciatus* (**Rawani *et al,* 2009**).

Petroleum ether extracts of *Piper longum* exhibited higher larvicidal activity against *C.quinquefasciatus* than *Curcuma aromatica*, and *Ocimum basilicum* (**Madhu and Vijayan, 2010**). A combination of *Clerodendron inerme* and *Acanthus ilicifolius* at different concentrations on *C.quinquefasciatus* showed significant increased mortality under laboratory and field conditions (**Kovendan and Murugan 2011**). Methanol and acetone

extracts of *Calotropis procera*, *Tephrosia purpurea* and *Prosopis juliflora* were found to be more effective against third and early fourth instars of *C.quinquefasciatus* than petroleum ether and aqueous extracts (**Bansal *et al,* 2012**).

2.3.7.3 Larvicidal activity of *E. crassipes*

Putative cytokinin glucoside-like activity was detected only in leaves and flowers. The cytokinin complements of the leaves and the roots were qualitatively different. Cytokinins supplied by the roots are metabolized in the leaves, and certain cytokinins are synthesized in the leaves themselves (**Nagar and Saha, 1985**). The acetone extract had moderate feeding deterrent and toxic activities towards third instar larvae of two lepidopteran pests, the tobacco cut worm, *Spodoptera litura* and the castor semilooper, *Achaea janata* (**Devanand and Usharani, 2008**). *Chironomus ramosus* Chaudhuri eggs and larvae subjected to varying concentrations of crude root extracts of *E. crassipes* (final concentrations 0.25-2.5%) showed 100% efficiency (**Thorat and Nath, 2010**). The aqueous extract of *E. crassipes* tested against the second instar larvae of *Culex pipiens* and the resulting pupae and adults at 2% concentration revealed drastic effect on larval midgut, integument, fats and muscles (**Assar and El-Sobky, 2003**).

2.4 *In silico* docking studies

Drug discovery process is a critical issue in the pharmaceutical industry since it is a very cost-effective and time consuming process (**Recanatini *et al,* 2004; Bharat *et al,* 2011**) to produce new drug potentials and enlarge the scope of diseases incurred. Sophisticated *in silico* approaches has given a tremendous opportunity to pharmaceutical companies to identify new potential drug targets which in turn affect the success and time of performing clinical trials for discovering new drug targets (**Rao and Srinivas, 2011**). These *in silico* methods include databases, quantitative structure-activity relationships, similarity searching, pharmacophores, homology models and other molecular modeling, machine learning, data mining, network analysis tools and data analysis tools that use a computer. Such methods in recent years have seen frequent use in the discovery and optimization of novel molecules with affinity to a target, the clarification of absorption, distribution, metabolism, excretion and toxicity properties as well as physicochemical characterization (**Ekins *et al,* 2007**).

2.4.1 Software-*Hex*

Hex was written by Dave Ritchie at the University of Aberdeen. The main thing which distinguishes *Hex* from other macromolecular (i.e. protein and DNA) docking programs and molecular graphics packages is its use of *polar Fourier correlations* to accelerate docking calculations. The graphical nature of *Hex* came about largely because it can be used to visualise the results of such docking calculations in a natural and seamless way, without having to export unmanageably many (and usually quite big) coordinate files to one of the many existing molecular graphics packages (**Ratna and Sheeba, 2008**).

2.4.1.1 Docking studies carried out using *Hex* software

Rigid-body docking studies of HIV-1 RT (Brookhaven Protein Data Bank accession nos: 1HMV, 1RTH, 1RTD and 1HVU) and *Hex*-S3 were performed with the program *Hex* 4.2. *Hex*-S3 binds the p66 subunit at the bottom of the thumb domain pointing towards the nucleic acid binding cleft of the viral polymerase. Binding of this hexanucleotide ligand requires the thumb domain to be in an upright position (**Mescalchin *et al,* 2006**).

The interaction of pres1 region of hepatitis B virus B-cell epitope antigen with specific hepatitis B neutralizing monoclonal antibody was examined by docking study using *Hex* 4.2 software. It was found that the docked complex is stabilized by 59·3 kcal/ mol. The stability of the docked antigen-antibody complex is due to hydrogen bonding and van der Waals interactions (**Rajkannan and Malar, 2007**). The acetone extract of whole part of *Elephantopus scaber* yielded a new terpenoid which was analysed for its protein-ligand interactions with autolysin using Hex docking software. The docking score with minimum energy value of -209.54 was calculated which showed that the novel terpenoid possesses antibacterial activity (S.aureus) by inhibiting autolysin (**Daisy *et al*, 2008**).

Human estrogen receptor (2IOK) and the commercially available drugs like Tamoxifen, Raloxifene, Toremifene against breast cancer were analysed for its molecular interactions using *Hex* and the energy value obtained was lower for Tamoxifen (**Mathew and Raj, 2009**). Molecular docking between β-ketoacyl-acyl carrier protein synthase (KAS) 1HNJ receptor and designed benzimidazole derivatives containing isoxazole moiety carried out using *Hex* docking software showed good binding energy when compared to the standard suggesting the designed compounds to be potent antibacterial agent (**Kumar *et al,* 2010**).

The energy difference in the docking study by separate batch wise docking of leptin with receptor, leptin-receptor complex with Mahanimbine using *Hex* software version 6.3 showed that Mahanimbine is more strongly associated with leptin in complex form rather than isolated form with maximum energy score indicating that Mahanimbine may be used as combination with leptin to treat obesity patient (**Ashokan, 2011**).

Miglitol, D-pinitol, 3-hydroxymethyl xylitol and few other cyclitols have been docked as ligands to DPP4 using *Hex* 6.1 docking software. The compound pinitol diacetonide was found to be more compatible with DPP4 compared to the other compounds studied as evident from its E value of -200.62. The cyclitols have been computed to be good anti diabetic agents (**Lalitha and Sripathi, 2011**). Brain-derived neurotrophic factor (BDNF) (1BND), which co-exist with Type II Diabetes and Ras homolog gene (RHOD) (1JIL) were docked against antioxidant ligands *Astaxanthin* and *β-carotene* using *Hex,* which *Astaxanthined* a lower energy score when compared with *β-carotene* is lowest which showed the highest affinity with the target proteins (**Guttala *et al,* 2011**). The receptors 1DFB, 2EBO, 2X7R, 1AIK, 3EGS, 3MNW and 1CVU were docked with different pyrrole derivatives. The results obtained showed that the iodo pyrrole derivative has high interaction energy compared to other fluoro and methyl derivatives (**Patel *et al,* 2012**).

2.4.2 Review pertaining to the docking of COX-1 and COX-2

A group of celecoxib analogues in which the para-SO_2NH_2 substituent on the N^1-phenyl ring was replaced by a *para*-sulfonylazido (SO_2N_3), or a meta-SO_2N_3 substituent were designed for evaluation as selective cyclooxygenase-2 (COX-2) inhibitors. Docking studies was performed using Insight II software (Version 97) running on a Silicon Graphics Indigo 2 workstation. An appropriately positioned SO_2N_3 moiety acted as a novel alternative bioisostere to the traditional SO_2NH_2 and SO_2Me pharmacophores present in selective COX-2 inhibitors (**Uddin *et al,* 2003**).

Molecular docking for a set of eighty two structurally diverse COX-1/COX-2 inhibitors including traditional NSAIDs and the recently developed coxibs was carried out using FlexX method. Visualization of docked inhibitors in COX-2 enzyme revealed that the carboxylate group of NSAIDs is located in a favourable position to interact with the guanidinium group of Arg120 and OH of Tyr355 (**Chakraborti and Thilagavathi, 2004**).

A set of thirty-four 1,5-diaryl pyrazoles having selective COX-2 inhibitory activity were analyzed using Comparative Molecular Field Analysis (CoMFA) and Comparative

Molecular Similarity Indices Analysis (CoMSIA) in two different alignments. Compared to the alignment method involving the docked conformations, the atom-based alignment produced better CoMFA and CoMSIA results (**Thilagavathy and Chakraborti, 2005**).

Self-organizing molecular field analysis (SOMFA), has been used to study the correlation between the molecular properties and the anti-inflammatory biological activities of a new series off, 5-Diarylimidazoles that act as selective COX-2 inhibitors. The statistical results, cross validated rCV^2 (0.507) and non cross-validated r^2 (0.546), showed a satisfied predictive ability (**Li and Zheng, 2006**).

The docking experiment of 3, 4-diaryl- 2(5*H*)furanones was performed using Insight II software version. A molecular modelling study showed that the *N*-acetylsulfonamido compound bound in the centre of the primary COX-2 binding site such that the C-4 *para*-SO_2NHCOMe substituent is oriented, like the C-4 *para*-SO_2Me moiety in rofecoxib, in the vicinity of amino acid residues lining the COX-2 secondary (2°) pocket (Ala516, Ile517, Phe518, Arg513 and Gln192) (**Zarghi *et al,* 2007**).

Molecular docking experiments were carried out using Autodock v4.0.1 to identify potential COX-2 inhibitors among the β-hydroxy-β-aryl-alkanoic acids class into the 3D structure of the catalytic site of COX-2 enzyme (pdb code: 1cx2) and COX-1 enzyme (pdb code: 1eqg) and it was found that three class of compounds possess the strongest anti-inflammatory activity, comparable to that of ibuprofen (**Dilber *et al,* 2008**). A set of 5-phenyl-1-(3-pyridyl)-1*H*-1,2, 4-triazole-3-carboxylic acid derivatives showing anti-inflammatory activity were docked into COX-1 and COX-2 X-ray structures, using the program GOLD, which suggested the effective activity of novel triazole derivatives against COX inhibitors with a significant preference for COX-2 (**Linder *et al,* 2009**).

Benzoimidazles were designed based on the structure of receptor, COX-II and well known NSAID Celecoxib. Several of the docked ligands were found to have less binding energy in kJ/ mole and said to possess more affinity for receptor than other molecules and celecoxib (**Chhajed *et al,* 2010**). The anti-inflammatory compounds of piroxicam, meloxicam, tenoxicam, isoxicam and the natural α- and β-cyclodextrin structures have been studied by MM+ program from the HyperChem 5.1, a RMS of 0.05 kcal/mole and a Polak-Ribiere algorithm were used in the molecular modelling process. For both structure

types the most stable conformations were used in *host-guest* interaction analysis (**Hadaruga, 2011**).

Ethyl 5-(3(4- chlorophenyl) ureido-1H-1, 2, 4-triazol-3-acetate was docked against COX-2 and COX-1 enzymes by Molecular Operating Environment as the computational software using (PDB ID: 4COX) and (PDB ID: 1CQE) for COX-2 and COX-1, respectively the docking top ranking score for COX-2 was -12.365 kcal/ mole and for COX-1 was -11.668 kcal/ mole (**Wahab *et al,* 2011**). A series of novel acrylic acid derivatives bearing at the 3 position thienyl, furfuryl and 3, 5-ditert-butyl-4-hydroxyphenyl substituents have been tested as potential cyclooxygenase-1 inhibitors with GOLD and docking poses were obtained by applying both Chemscore and Goldscore, fitness functions available for scoring (**Pontiki *et al,* 2011**).

Docking studies were carried out using GOLD software to determine the inhibitory effect of few compounds as COX-2 inhibitors. Bisdemethoxy curcumin, Yukechenone A, Capsaicin and paradol showed highest binding affinity for COX-2 of *Homo sapiens* (**Shruthi *et al,* 2012**). Cyclooxygenase inhibitory activities of flavonoids like Naringenin, Celecoxib etc., were evaluated *in silico* using AutoDock 4.2. All the selected flavonoids showed binding energy ranging between -8.77 kcal/ mol to -6.24 kcal/ mol when compared with that of the standard (-8.30 kcal/ mol) and its activity correlated with its structural parameters (**Madeswaran *et al,* 2012**).

2.4.3 Literature pertaining to the use of *Hex* software for docking cyclooxygenases

Out of ten ligands chosen to dock with the receptor molecule, cyclooxygenase- 2 using *Hex*, Gingerol with a maximum of 5 hydrogen bonds was found to be the best inhibitor molecule for cyclooxygenase-2 enzyme. The energy minimization value obtained showed that curcumin has a minimum E value of -241.1 which showed that curcumin is also a best inhibitor for cyclooxygenase enzyme followed by capsaicin, ketorolac, naproxen, with a maximum of 6 and 5 hydrogen bonds (**Rathna and Sheeba, 2008**).

2.4.4 SIRT1

Sirtuin isoforms contribute to various key aspects of metabolic regulation, disease pathologies, and ageing. They are thus considered attractive therapeutic targets for diseases such as cancer and neurodegenerative disorders which have spurred interest in

the mechanisms of Sirtuin catalysis and regulation and in small-molecule regulators for *in vivo* studies and therapy (**Schlicker *et al*, 2011)**.

2.4.4.1 Literature regarding the docking of SIRT1

Computational docking analysis showed that Sirtinol and Salermide have high degrees of selectivity for SIRT1/2, whereas EX527 has high specificity for SIRT1 but not SIRT2 (**Peck *et al*, 2010**). Investigation of promoter DNA methylation and gene expression of highlighted by the *in silico* analysis in response to SIRT1 manipulation, revealed that changing the level of SIRT1 expressed in Caco-2 cells for six of the eight genes resulted in a significant change at atleast one of the CpG sites captured in the assay and also had an effect on the expression, at the mRNA level, of all eight of these genes (**Ions, 2011**). SIRT1 gene codes for the protein 'SIRTUINS' by using Bioinformatics Tools & Softwares Exons, Introns, SNPs and RFLPs of SIRT1 were predicted. The 3D structure of Sirtuin was modeled and docked with suitable ligands. The SIRT1 activation study helps in medical application for developing SIRT1 activating drugs for ageing problems (**Mahendran *et al,* 2011**).

A 3D comparative model of the human SIRT1 enzyme was predicted by the PHYRE server using a yeast Sir X-ray crystal structure (PDB entry code: 2HJH, chain A) as template. 2HJH showed high sequence identity with the target protein and high Blast and SAS scores (**Fruscia *et al,* 2012).**

Resveratrol and human Sirtuins (1 to 6) were docked against Type 2 Diabetes mellitus HNF-1a motif with PDB ID: 2GYP using Hex 5.1. Resveratrol and Sirtuin 6 have good energy score amonst other ligands (Kaladhar, 2012).