4.0 DISCUSSION

Medicinal plants are important source of secondary metabolites with interesting medicinal properties. They constitute the main source of new pharmaceuticals and healthcare products (Ivanova et al., 2005). The secondary plant metabolites play critical roles in human health and may be nutritionally important (Hertog et al., 1993). The most important of these secondary metabolites are alkaloids, tannins, flavonoids, phenolic compounds, and saponins (Garro, 1986). *Cissampelos pareira* L var. hirsute belongs to the family Menispermaceae was selected for the study based on the ethno botanical information compiled through interviews from siddha and ayurveda medical practitioners. This plant is used as a cure for menstrual problems, prevent miscarriage, and control uterine hemorrhages, hormonal acne premenstrual syndrome etc.

*Cissampelos pareira* L var. hirsuta (Buch.-Ham.ex DC.) Forman is a perennial twining shrub, Leaves acuminate with an apicula at the apex. The stem is flexible, and slender up to 1cm and twines for support. The root system consists of flexible, light brown, lateral roots with sinkers and moderately abundant fine roots. Male flowers are pale yellow, female racemed, drupes are orange to red with one seed. Seeds are horseshoe-shaped; embryo elongate, narrow, embedded in endosperm, cotyledons flattened. Flowering in the month of April-May and fruiting in the month of June.

DNA barcoding is a new concept used for the identification, discrimination or taxonomic classification of species using standardized DNA sequences as tags. It is used for the molecular identification of already described species (Hebert et al., 2003)
and in the discovery of new species (Valentini et al., 2006). It is a method of choice that uses a short DNA fragment as a tag, to define or to discover a species. The consortium of the Barcode of Life PlantWorking Group has identified a few locias potential barcode candidates and from them a two locus standard barcode (\textit{matK} and \textit{rbcL}) has been recommended for initiating the barcoding process of plant species (Kress et al., 2005). DNA barcoding uses minor differences of nucleotides in the particular gene loci of different organisms as a key for the discrimination. The gene is sequenced to know the base-pair differences and then deposited in the barcode database, which is termed as DNA barcodes. These genetic codes could be accessed through a digital library and used to identify the unknown species by any scientist around the world. In DNA barcoding, short DNA fragments (400-800 bp) are used as specific reference collections to identify specimens and to discover overlooked species (Savolainen et al., 2005). The main reason for DNA Barcoding in species is to gain biological identification, determine evolutionary and phylogenetic relationship and finally to preserve its molecular data in an electronic library of barcodes.

BLAST is an effective and most cited method for identification (www.ncbi.nlm.nih.gov/BLAST/) of species based on the best sequence alignment (Kent, 2002). Simple measures of genetic distance have commonly been used in barcoding studies to infer identity, with thresholds used to distinguish non-specifics from heterospecifics (Hajibabaei et al., 2007). MEGA tool is used to find the evolutionary relationships between the species using homologous sequence. It is based on the statistical analysis of genes, the percentage of conservedness, variance and parsimony of the sequences. The distance between the sequence pair, average
distance within and between groups also can be estimated which was accomplished using bootstrapping approach. Transition and transversion type of substitution between the sequences can also be estimated. It is used for inferring phylogenies by the distance-based methods, along with the bootstrap test. This tool is used for, estimating evolutionary distance, constructing phylogenetic trees, testing tree reliability, marking genes and domains, testing for selection, grouping sequences, computing sequence statistics, constructing trees from distance data.

In the present investigation, the genomic DNA was isolated by the method of Doyle and Doyle (1987) from the seven samples of *C. pareira* collected from different ecotypic regions such as Ambasamudram (AC1), Ooty (AC2), Peermade (AC3), Kumaracoil (AC4), Kozhikode (AC5), Yelahanka (AC6) and Ariyankuppam (AC7) and the PCR amplicons for barcode candidates *matK* and *rbcL* were resolved using the agarose gel. In this study, *rbcL* and *matK* plant barcode were used to provide the DNA sequence frame work of seven samples. The evolutionary divergence between the sequences estimated a total of 842 basepairs for *matK* and 697 basepairs for *rbcL* with 100% match except for the sample AC6. The intra-specific genetic divergence of each sequence of the samples AC1-AC7 of *matK* and *rbcL* showed no intra-specific divergence expect for AC6 with 1% variation. The overall intra-specificity among the *matK* and *rbcL* were estimated as 0.001 and 0.00014 respectively. In consonance with the present investigation, Ma et al. (2014) and Vassou et al. (2015), who reported that the inter-specific sequence divergence among DNA barcode regions was highest in *matK* and *rbcL*, in *Sida cordifolia* and *Tulipa edulis*. 

68
Phylogenetic analysis is used to determine the evolutionary relationships between species. It defines the inter, intra-generic relationship. It is an attempt to discern the ancestral relationship of a set of sequences. It involves the construction of a tree, where the nodes indicate separate evolutionary paths, and the lengths of the branches give an estimate of how distantly the sequences are related. The results of an analysis can be drawn in a hierarchical diagram called a cladogram or phylogram (phylogenetic tree). The branches of a tree denote the hypothesized evolutionary relationships (phylogeny). Each member in a branch, also known as monophyletic group assumed to be descended from a common ancestor. Originally, phylogenetic trees were created using morphology and now it is carried out using the DNA sequences (Saitou and Nei, 1987). In the present study, the phylogenetic tree was constructed using maximum likelihood nearest neighbor interchange method and the results clearly revealed that matK and rbcL regions of the sample DNA, namely AC6 could distinguish among the plant species of the family. Thus phylogenetic study revealed the evolutionary history of Cissampelos pareira among the genus and also with the closely related species. The study also proved that matK and rbcL as DNA barcode candidates could help in categorical identification of C. pareira. Ortiz and Nee (2014) reported that Cissampelos sp. are monophyletic and their relationships within their subtribe were still unclear. The slight diversion in their gene level leads due to variation in their sepal and anteseptalous petal of the flower. The matK and rbcL sequencing method showed good amplification efficiency in species discrimination within the family of Cissampelos sp. Hollingsworth et al. (2011) reported DNA barcoding in several plant species, through sequence analysis and the results reflected the general statement that the regions based on nuclear DNA are
highly specific when compared to barcode regions from organelle DNA which were in agreement with the present study. The present investigation corroborated with the findings of Selvaraj et al. (2008), who reported that the Zingiberaceae genus shows polyphylogeny in the study of sequence alignment of matK gene sequences obtained from genbank by Clustal X, transition, transversion rates by MEGA and the phylogenetic analysis by PHYLIP.

In the present investigation of the computational study on the translation expression profile of seven samples of *C. pareira*, DNA barcoding sequence reflected their unique molecular homology characters in their three letter codon region frequency except for *rbcL* barcoding sequence of *C. pareira* and the sample AC6 showed slight variation in aminoacid profile such as GAT, ATT, CAA when compared to other barcoding sequences. The present results supports the findings of Group et al. (2009), who reported that the homology among the protein expression profile which explore the common ancestral residues function of the species share the unique molecular functional informations. Thus the overall results of the evolutionary divergence, phylogenetic and the molecular expression profile makes a solid conclusion on their common molecular functions. Since the codon and the related amino acid expression were shown similarity among the seven collected samples which revealed their common molecular functions, hence *C. pareira* (AC4) collected from Kumaracoil, Kanyakumari district, was chosen for further phytochemical and other pharmacological studies. In view of the medicinal properties attributed to different parts of the selected plant, phytochemical investigation has been carried out in leaf, root and stem using various solvents.
Plants are important source of chemotherapeutic agents, hence phytochemical analysis is of paramount importance in identifying new sources of therapeutically and industrially valuable compounds (Ambasta, 1986). Plants that are endowed with molecules such as vitamins, terpenoids, phenolic acids, tannins, flavonoids, quinones, coumarins, alkaloids and other metabolites which are responsible for the antioxidant and free radical scavenging activity (Aiyegoro et al., 2009; Zheng and Wang, 2001 and Cai et al., 2004). Studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumour, antimutagenic, anticarcinogenic, antibacterial, and antiviral activities (Sala et al., 2002 and Rice-Evans et al., 1996).

In the present study, hexane, chloroform, ethyl acetate ethanol and aqueous extracts of leaf, root and stem of C. pareira revealed the presence of alkaloids, flavonoids, tannins, phenols, steroids, terpenoids, saponins, cardiacglycosides, aminoacids and volatile oil. Among the various extracts and different parts of C. pareira, the ethyl acetate leaf extract showed the presence of more phytoconstituents and the results were in conformity with the findings of Gagandeep Kaur et al. (2016), who reported the presence of alkaloid, flavonoid, tannins, steroids,phenols and saponins during the phytochemical screening of hexane, chloroform, ethylacetate and methanolic leaf extracts of Tiliacora triandra belonging to the family Menispermaceae, where the ethyl acetate and hexane extract showed the presence of more phytoconstituents. Likewise, Rabari et al. (2010) reported the presence of various phytochemicals such as alkaloids, carbohydrates, polyesterols and proteins during the phytochemical screening of chloroform and ethylacetate leaf extracts of Cocculus pendulus belonging to the family Menispermaceae, where the ethyl acetate extract showed the presence of more phytoconstituents.
Phytochemical analysis conducted on various plant extracts revealed the presence of phytoconstituents which are known to exhibit therapeutic as well as physiological activities (Sofowora, 1993). The analysed bioactive compounds in the present study have a broad range of biological activities. Alkaloids are associated with cytotoxic property (Nobori et al., 1994), antispasmodic and antibacterial property (Okwu and Okwu, 2004), anti-inflammatory and antioxidant property (Banu et al., 2010). Flavonoids possess a wide range of therapeutic uses such as antioxidant, anti-inflammatory, anti-mutagenic, anti-carcinogenic, free radical scavenging activities and also decrease cardiovascular complications (Trease and Evans, 2002; Khan and Sultana, 2006). Tannins have been reported to prevent the development of microorganisms by precipitating microbial protein (Prasad et al., 2008). They are used in the treatment of burns as they precipitate the proteins of exposed tissues to form a protective covering (Handa and Kapoor, 1989). They are also used as healing agents in inflammation, leucorrhoea, gonorrhea and in piles (Ali and Storey 1994). They are known to possess anti-diarrhoea properties (Mbaebie et al., 2012).

Plant phenols constitute the major group of compounds that act as primary antioxidant Hatano et al. (1989). It also possesses anti-inflammatory, anti-carcinogenic, anti-atherosclerotic and antioxidant activities (Chung et al., 1998; Khoobchandani et al., 2012). Phenolic compounds are receiving increasing attention because of their health promoting effects (Meot and Magne, 2009; Chahal et al., 2011; Nakamura et al., 2003). Steroids are reported to have cholesterol-reducing properties. It helps in regulating the immune responses (Shah, 2009). They are also reported to have antibacterial properties (Raquel, 2007). Saponins are hypoglyceamic, antifungal and antihyperlipideamic agents in animals (Desai et al., 2009). They play a prominent role
in ensuring hormonal balance and synthesis of sex hormones (Okwu, 2005) which is also related to reduce cancer risk (Thompson et al., 1993). Cardiac glycosides has an important role in medicine because of their action on heart and used in cardiac insufficiency (Swedberg et al., 2005). They are naturally cardio active drugs used in the treatment of heart failure and cardiac arrhythmia and also to lower the blood pressure (Potpara et al., 2016).

The results obtained in the present study suggested that the selected plant was proved to be an increasingly valuable reservoir of bioactive compounds. The preliminary phytochemical tests are helpful in finding chemical constituents in the plant material that may lead to their quantitative estimation and also in locating the source of the pharmacologically active chemical compounds.

Thin layer chromatography is an important analytical tool for the separation, identification and estimation of different classes of natural products (Hultin, 1965). TLC optimization of the solvent system for development and purification of secondary metabolites or other compounds from plants is a very crucial step (David et al., 1982). In the present investigation, thin layer chromatography was carried out in various extracts of different parts of *C. pareira* such as leaf, root and stem with solvents such as hexane, chloroform, ethyl acetate, ethanol and aqueous using hexane : ethyl acetate : acetic acid (5 : 4 : 1) as mobile phase. TLC analysis resulted in the formation of spots which indicated that the mobile phase was suitable to separate the phytocompounds in plant extracts with good resolution.

TLC analysis of leaf extracts of *C. pareira*, resulted in the identification of 10 spots in ethyl acetate extract, 4 spots in ethanol, 3 spots in hexane and aqueous
extract, whereas that of root extracts, resulted in the identification of 4 spots in ethyl acetate extract, 3 spots in chloroform extract, 2 spots in aqueous extract and 1 spot in hexane and ethanol extract. In the case of stem extracts, the analysis resulted in the identification of 5 spots in ethanol extract, 3 spots in ethyl acetate extract, 2 spots in aqueous and hexane extract and 1 spot in chloroform extract. TLC showed that the phytochemicals present in varying quantities in different extracts which are evident from the number of spots with different $R_f$ values. The present results revealed that the ethyl acetate leaf extract showed maximum number of spots corresponding to more number of phytoconstituents. Hassan et al. (2015) reported the TLC analysis of chloroform, ethyl acetate and petroleum ether leaf extracts of *Senna siamea* and revealed that among the three extracts ethyl acetate and chloroform showed 9 spots each and petroleum ether extract showed 2 spots. Thin layer chromatographic study by Swathi (2016) in different leaf extracts of *Moringa Olifera* and revealed that ethyl acetate showed maximum number of spots among different extracts with different $R_f$ values using the solvent system hexane: ethyl acetate: acetic acid (5:4:1). Thus the TLC profiling of *C. pareira* gave a clear evidence that directs towards the presence of number of phytochemicals with different $R_f$ values in different solvent system. The variation in $R_f$ values of the phytochemicals provides a very important clue in understanding the polarity and also helps in selection of appropriate solvent system for the separation of pure compounds by column chromatography and hence a mixture of solvents with variable polarity in different ratio can be used for separation of pure compound from plant extract. The selection of appropriate solvent system for a particular plant extract can only be achieved by analysing the $R_f$ values of compounds in different solvent system.
Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the selected plant extracts (Cook and Samman, 1996). Phenolic compounds have redox effects, which allow them to behave as antioxidants (Soobrattee et al., 2005). As their free radical scavenging ability was facilitated by their hydroxyl groups, the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity. Flavonoids are also relatively powerful scavengers of most oxidizing molecules, such as singlet oxygen, and various other loose radicals implicated in numerous diseases. Flavonoids suppress the reactive oxygen formation, chelate trace elements involved in free-radical production, scavenge reactive species and protect antioxidant defenses (Agati et al., 2013).

In the present study, the total phenolic and flavonoid content of different extracts of *C. pareira*, calculated from the calibration curve ($R^2 = 0.997$) ranged from 12.52 ± 1.09 to 47.76±1.09 mg GAE/g and 36.19 ± 1.73 to 62.3±1.27 mg QE/g respectively. Mir Zahoor et al. (2016) studied the total phenolic content ranged between 2.44±0.05 to 10.14±0.14 mg GAE/g and the flavonoid content ranged between 6.92±0.19 and 28.49±0.75 mg QE/g in different leaf extracts of *C.pareira* which were in conformation with the present study. The present results were also comparable with the findings of Rattana et al. (2010), who reported that the flavonoid content in the leaf extracts of *Tiliacora triandra* measured by the aluminum chloride colorimetric assay was found to be 18.67±0.28 mg QR/ g of extract. The phenolic and flavonoid content, present in the plant parts are widely used in combating oxidative stress related diseases (Chen et al., 2014 and 2015).
Gas Chromatography Mass spectrometry, a hyphenated system is a very compatible and most commonly used technique for the identification and quantification of phytoconstituents. The unknown phytocompounds in a complex mixture can be determined by interpretation and by matching the spectra with reference spectra (Hites, 1997). The more precise information in qualitative analysis can be obtained by gas-chromatography coupled with spectrometry (GC-MS) (Zhang and Zuo, 2004).

In the present study of the GC-MS analysis, the ethyl acetate leaf extract of *C. pareira* revealed the presence of eight compounds. Two constituents namely 17-Pentatriacontene (C$_{35}$H$_{70}$), mw. 490 and 3,7,11,15-Tetramethyl 2 hexadecen-1-01 (phytol) C$_{20}$H$_{40}$O, mw.296 were found to be the major components at RT 52.65 and 31.95 with 28.30% and 24.90% peak area respectively. The compound 17-Pentatriacontene is a hydrocarbon having antibacterial activity whereas the compound phytol is known to be antimicrobial, antioxidant, anticancer, anti-inflammatory and hepatoprotective. The other constituents identified are n-Hexadecanoic acid (RT 34.41) having antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant, and anti-inflammatory activity, 1,2- Benzenedicarboxylic acid, disooyctyl ester (RT 44.33) have antimicrobial and antifouling activity, Ergost-5-en-3-ol, acetate (RT 51.09) have antiangiogenic, antiflu, antitumour and antiviral property, 1,30-Triacontanediol (RT51.47) and Stigmasta (RT 51.64) have antihepatotoxic, antioxidant, hypocholestrolemic anti-inflammatory, estrogenic, and antiviral activity.17-Pentatriacontene, which was the major component revealed in the present study was reported by Tayade et al. (2013) in the root extracts of *Rhodiola imbricate*. Similarly another major component phytol in the current study was reported by
Abdelhamid et al. (2015) in the ethanolic seed extracts of *Nelumbo nucifera* and Asish et al. (2013) in the ethyl acetate leaf extracts of *Lagassea mollis*. Similarly, Rani et al. (2011) observed the presence of phytol in the leaves of *Lantana camara* and Sridharan et al. (2011) in *Mimosa pudica* leaves. The present study also correlated with the findings of Papitha et al. (2016), who reported the presence of compound such as 3, 7, 11, 15-Tetramethyl 2-Hexadecen-1-ol in the methanolic stem extract of *Tinospora cordifolia*, which possessed analgesic, anti-inflammatory and antipyretic activities.

Therefore GC-MS method is a direct and fast analytical approach for identification of bioactive components. The present study which revealed the presence of components in ethyl acetate leaf extract of *C. pareira* suggested that the contribution of these compounds on the pharmacological activity should be evaluated. The investigation proved that the stronger extraction capacity of ethyl acetate could have been produced number of active constituents responsible for many biological activities, which can be utilized for the development of traditional medicines and further investigation needs to elute novel active compounds from the medicinal plants to treat many incurable diseases.

Herbs and herbal products are reported to have antibacterial potential (Adwan et al., 2006) and hence herbal treatments become very popular and it is easily available, cheaper and less toxic than synthetic drugs. In the present investigation, the hexane, chloroform, ethyl acetate, ethanol and aqueous extracts of leaf, root and stem of *C. pareira* were evaluated for exploration of their antibacterial activity against three pathogenic gram positive bacteria such as *E. faecalis, S. aureus, S. pyogenes* and
three gram negative bacteria such as *V. cholerae*, *K. pneumoniae*, and *S. typhimurium*. Susceptibility of each plant extract was tested by agar-well diffusion assay and MIC and MBC values were determined. In the case of leaf extracts, the ethyl acetate exhibited maximum growth inhibitory activity of 20.96±0.95 mm and 20.80±0.72 mm against *K. pneumoniae* and *S. typhimurium*, whereas chloroform extract exhibited minimum activity (05.16±0.76 mm) against *E. faecalis*. In the case of root extracts, the ethyl acetate extract exhibited maximum growth inhibitory activity of 15.56±0.51 mm against *S. aureus* and ethanol extract exhibited growth inhibitory activity of 14.56±0.51 mm against *K. pneumonia*, whereas aqueous extract exhibited minimum inhibitory activity (05.10±1.25 mm) against *S. aureus*. In the case of stem extracts, the ethyl acetate extract exhibited maximum growth inhibitory activity of 10.83±1.04 mm against *K. pneumoniae* and ethanol extract exhibited growth inhibitory activity of 10.53±0.50 mm against *S. pyogenes*; whereas chloroform extract exhibited minimum inhibitory activity (05.13±1.02 mm) against *Streptococcus pyogenes* and 05.16±0.50 mm against *K. pneumoniae*. In consonance with the results of the present study, Anowi *et al.* (2012), who reported that the ethyl acetate leaf extracts inhibited all the bacteria in a most appreciable extent in the study of various leaf extracts of *Ritchiea longipedicellata* against gram positive and gram negative bacteria such as *S. aureus*, *P. aeruginosae*, *Klebsiella* sp., *E. coli*, *B. subtilis*, *S. lutea*, *S. typhimurium* at various concentrations ranging from 6.25 to 200 mg/ml. Shanthi and Nelson (2013) reported that the ethanol leaf extract of *T. cordifolia* showed greater inhibitory action against *K. pneumoniae* followed by *P. aeruginosa* among other tested extracts by agar well diffusion method.
Further, the effectiveness of hexane, ethyl acetate and ethanol leaf extracts of *C. pareira* were determined by agar- disc diffusion method at three different concentrations such as 1.25, 2.5 and 5 mg/disc against three pathogenic gram positive bacteria such as *E. faecalis*, *S. aureus*, *S. pyogenes* and three gram negative bacteria such as *V. cholerae*, *K. pneumoniae* and *S. typhimurium* and was compared with standard antibiotic Streptomycin. The results showed that at different concentrations, crude leaf extracts of *C. pareira* strongly inhibited the growth of all test bacterial strains. The ethyl acetate extract at a concentration of 5mg/disc exhibited maximum growth inhibitory activity of 15.50±0.50 mm and 15.47±0.21 mm against *S. pyogenes* and *S. typhimurium*. The present study was in agreement with the findings of Shahadat Hossain (2014), who revealed that the highest zone of inhibition (18 mm) was found in the concentration of 200µg/ disc for *Klebsiella* species in the ethyl acetate leaf extract of *Ficus benghalensis* against gram positive and gram negative bacteria by disc diffusion method.

MIC and MBC are used to assess the antimicrobial efficacy of metabolites or synthetic compounds. In the current study, the ethyl acetate extract has shown very low MIC value (15.6 µg/ml) against *S. aureus*, *S. pyogenes* and *K. pneumoniae*. This showed that the extract has good antibacterial activity. Further the antimicrobial activity of plant extracts were strengthened by low MBC values obtained in the plant extracts against the bacterial strains. However, the low MBC value was obtained in ethyl acetate extract of *C. pareira* against *K. pneumoniae* and *S. aureus* (31.25 µg/ml). The MIC and MBC values were compared with the standard broad spectrum antibiotic streptomycin. The present results was in conformity with the findings of Sivaperumal *et al.* (2010), who reported that the MIC value (1.80 µg/ml) and MBC value
(2.05 µg/ml) showed highest activity in ethyl acetate extract against *S. aureus*, *S. pyogenes*, *K. pneumonia*, *S. typhimurium* and *P. aeruginosa* among various leaf extracts of *Aplidium multiplicatum*.

From the present work, it can be stated that the ethyl acetate leaf extract of *C. pareira* was found to be more potent and showed equal antimicrobial effects on both gram positive and gramnegative bacteria. It also showed the presence of strong antimicrobial constituents belonging to the different groups (Batista *et al.*, 1994 and Lambert *et al.*, 2001) which have very high permeability across the bacterial cell wall (Walsh *et al.*, 2003). It is suggested that based on present study, different phytochemicals revealed their presence in the plant extracts of *C. pareira* makes the plant potent against bacterial pathogens. There is growing interest in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity. Phytochemicals are non-nutritive plant chemicals that may have protective or disease preventive activities. Flavonoids, alkaloids and saponins are found to be associated with antimicrobial effects in various studies using plant extracts (Nwaogu *et al.*, 2007). Flavonoids have been found to exhibit antimicrobial activity through various mechanisms like inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function and energy metabolism (Cushnie and Lamb 2005). Saponins possess detergent like activity and might increase the permeability of bacterial cell membrane without destroying them (Arabski *et al.*, 2012). Thus the phytochemicals of *C. pareira* can be used for the development of herbal medicine which might have a stronger applications against infectious pathogens.
In the present study, the antifungal activity of *Cissampleos pareira* was studied using three fungal pathogens namely, *A. niger*, *A. fumigates* and *P. chrysogenum* and the antibiotic fluconazole (10µg/disc) was used as the standard. The result inferred that, ethyl acetate leaf extract showed maximum inhibitory activity of 11.63±1.55 mm against *A. fumigates* and 11.43±1.30 mm against *A. niger* while, the standard antibiotic fluconazole displayed pronounced antagonistic activity of 22.83±1.66 mm against *A. fumigates*. In the case of root extracts of *C. pareira*, the ethyl acetate extract showed maximum inhibitory activity of 6.96±1.36 mm against *A. fumigates* and the antibiotic fluconazole displayed pronounced antagonistic activity of 23.78±1.94 mm against *P. chrysogenum*, whereas the ethanolic stem extract showed maximum inhibitory activity of 10.16±1.41 mm against *A. niger* and the antibiotic fluconazole displayed 23.33±2.32 mm of antagonistic activity against *A. fumigates*. It was reported that, the cell walls of fungi are poorly permeable and consist of polysaccharides such as hitchin and glucan (Huneck, 1999) and this may be the reason for its resistance towards fungal strains. On the other hand, fluconazole displayed good antifungal activity against all the tested fungal strains at lower concentration than the crude extract with significant differences in inhibitory zones. The present study supports the findings of Mann *et al.* (2008), who reported the maximum zone of inhibition ranging from 10.5 mm to 19.2 mm in ethyl acetate extract against *A. niger*, *A. fumigatus*, *Penicillium* sp. among various leaf extracts of *Anogeissus leiocarpa* and *Terminalia avicennioides*. Jaideep Singh *et al.* (2012) reported that the maximum zone of inhibition at the concentration of 40µl was 24 mm in the ethyl acetate leaf extracts of *Andrographis paniculata* against *A. niger* among various extracts at different concentrations such as 10 µl, 20 µl and 40 µl. In the present
investigation, the ethyl acetate leaf extract of *C. pareira* displayed considerably high fungistatic (MIC) and fungicidal (MFC) activity against pathogenic fungal strains was considerably higher. The MIC of ethyl acetate leaf extract of *C. pareira* was 31.25 µg/ml against *A. fumigates* indicating strong antifungal potential, while fluconazole, the standard antifungal drug showed activity against all the fungal strains tested. The lowest MFC (62.5 µg/ml) was observed for ethyl acetate leaf extract of *C. pareira* against *A. fumigates* which proved to possess the highest fungicidal activity. The present study supports the earlier investigations of Lumpu *et al.* (2014), who reported that the minimum inhibitory concentration (MIC) of 31.25 µg/ml and maximum fungicidal concentration (MFC) of 62.5 µg/ml in various leaf extracts of *Acalypha wilkesiana* and *Ageratum conyzoides* exhibited good and efficient antifungal activity against *Candida albicans*. The herbal based phytotherapy have large therapeutic applications since they can have less side effects when compared with synthetic antimicrobials (Iwu *et al.*, 1999). Thus the antifungal activity of the plant parts studied, showed prominent activity against *A. niger*, *A. fumigates* and *P. chrysogenum* which are potent pathogens.

Antioxidants are plant - derived compounds which protect the cell from harmful effects of reactive oxygen species which can control free radical formation (Jayachitra and Krithiga 2012). Free radical is a molecule with an unpaired electron and is involved in bacterial and parasitic infections, lung damage, inflammation, cardiovascular disorders, atherosclerosis, aging and neoplastic diseases (Burdon *et al.*, 1994), autoimmune disorders like rheumatoid arthritis (Bashir *et al.*, 1993), and function as free radical scavengers, initiator of the complexes of prooxidant metals, reducing agents and quenchers of singlet oxygen formation (Andlauer and
Phenolic compounds and flavonoids, the major constituents of plants are reported to possess antioxidant and free radical scavenging activity (Christensen et al., 2011; Hatano et al., 1989; Duh et al., 1999; Yen and Duh 1993 and Rice-Evans et al., 1996). Therefore, the importance for natural antioxidants has increased in the recent years (Jayaprakasha and Rao 2000). In the present study, the antioxidant activity of ethyl acetate, ethanol and hexane leaf extracts of *C. pareira* were assessed using *in vitro* assays such as a DPPH radical scavenging assay, H₂O₂ radical scavenging assay, nitric oxide radical scavenging assay, superoxide radical scavenging assay and reducing power activity.

DPPH (2,2-diphenyl picryl hydrazyl) is a commercially available purple coloured free radical, which can react with DPPH and to form yellow colour di-phenylhydrazine. DPPH radical quenching ability by antioxidants is primarily because of their hydrogen-donating ability. Hence, DPPH finds applications in the determination of the radical scavenging activity of plant materials (Jun et al., 2004). In the present study, the highest DPPH scavenging activity was noticed at 100 µg/ml concentration in the ethyl acetate extract (76.66 ± 0.67%) and the standard ascorbic acid also showed maximum activity of 87.97 ± 1.66% at the same concentration. According to the results, established in this investigation, it was found that the plant extracts showed notable scavenging activities against DPPH model in a concentration-dependent manner, that is, the higher the concentration, the highest scavenging potential. The ability of samples to scavenge DPPH radical was measured on the basis of their concentrations providing 50% inhibition (IC₅₀). The IC₅₀ values of ethyl acetate extract (65.81 µg/ml) and ascorbic acid (51.06 µg/ml) were obtained using the linear regression equation. The present study supports the earlier investigation of
Hilaria et al. (2016), who reported that ethyl acetate extract has strong antioxidant activity with IC$_{50}$ value 57.89 µg/ml when compared to standard ascorbic acid (IC$_{50}$ 3.781 µg/ml) among various leaf extracts of *Uvaria rufa* Blumes assessed by DPPH free radical scavenging activity. IC$_{50}$ is a value indicating the magnitude of the concentration of a substance, which can reduce DPPH radicals by 50%. The smaller the IC$_{50}$ means higher antioxidant activity. The present results showed that the ethyl acetate extract had lower IC$_{50}$ value compared with other extracts so that the ethyl acetate extract has antioxidant properties which is higher than hexane and ethanol extracts.

H$_2$O$_2$ is a weak oxidising agent which can diffuse through cell membrane directly and reacts with Fe$^{2+}$ and Cu$^{2+}$ ions to form -OH radicals which initiated toxic effects (Aruoma, 1999). It is therefore biologically advantageous for cells to control the amount of H$_2$O$_2$ getting accumulated. Scavenging of H$_2$O$_2$ by the plant extract could be attributed to its phenolics which donate electrons to H$_2$O$_2$, thus reducing it to water. In the present study, the extract demonstrated hydrogen peroxide radical scavenging activity in a dose dependant manner. The highest scavenging activity was noticed at 100 µg/ml in the ethyl acetate extract (82.11 ± 1.76%) and the standard ascorbic acid also showed maximum activity of 94.37 ± 1.18% at the same concentration. The IC$_{50}$ value of ethyl acetate extract (46.19µg/ml) and ascorbic acid (34.67 µg/ml) were obtained using the linear regression equation. The present study supports the recent investigation of Venkanna Banothu et al. (2017), who reported that ethyl acetate extract has strong antioxidant activity with IC$_{50}$ value 163.82 µg/ml when compared to standard ascorbic acid (IC$_{50}$ 860.23µg/ml) among various leaf extracts of *Albizia odoratissima* assessed by hydrogen peroxide radical scavenging.
activity. The present results showed that the ethyl acetate extract had lower IC$_{50}$ value compared with other extracts which confirms that the ethyl acetate extract has antioxidant properties which is higher than hexane and ethanol extracts.

The generation of nitrite ions by the interaction of oxygen and nitric oxide produced due to sodium nitroprusside in an aqueous solution at physiological pH is the basic principle of nitric oxide radical scavenging activity (Jagetia et al., 2004). Reduced production of nitric oxide from scavenging of nitric oxide which acts against oxygen can be monitored at 546 nm. In the present study nitrite produced by incubation of solution of sodium nitroprusside in standard phosphate saline buffer at 25°C was reduced by extracts of *C. pareira*. This may be due to antioxidant principles in the extracts which compete with oxygen. In the present study, the extract demonstrated nitric oxide radical scavenging activity in a dose dependant manner. The highest scavenging activity was noticed in the ethyl acetate extract at 100 µg/ml (81.57 ± 1.64%) and the standard ascorbic acid showed maximum activity at 100 µg/ml concentration (88.41 ± 1.06%). The IC$_{50}$ values of ethyl acetate extract (38.32 µg/ml) and ascorbic acid (27.71 µg/ml) were obtained using the linear regression equation. In consonance with the present results, Jain et al. (2009), who reported that the ethyl acetate extract has strong antioxidant activity with IC$_{50}$ value (51.3 µg/ml) when compared to standard ascorbic acid (IC$_{50}$ 58.1µg/ml) among various leaf extracts of *Cassia auriculata* which was assessed by nitricoxide radical scavenging activity. The present results showed that the ethyl acetate extract had lower IC$_{50}$ value compared with other extracts so that the ethyl acetate extract has antioxidant properties which is higher than hexane and ethanol extracts. The present results suggested that this plant might be potent and posses novel therapeutic agents.
for scavenging of nitric oxide and the regulation of pathological conditions caused by excessive generation of nitric oxide and its oxidation product.

Overproduction of superoxide anion radical contributes to redox imbalance and associated with harmful physiological consequences. Superoxide anion is generated in PMS-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan (Patel and Patel, 2011). In the present study, the highest scavenging activity was noticed at 100 µg/ml concentration in the ethanol extract (89.22 ± 1.00%) and the standard ascorbic acid showed maximum activity of (95.39 ± 1.41%) at the same concentration. The IC50 values of ethanol extract (55.05 µg/ml) and ascorbic acid (32.98 µg/ml) were obtained using the linear regression equation. Thus the ethanol leaf extract effectively inhibited O2 dependent NBT reduction in a concentration-dependent manner than hexane and ethanol extract, thus, indicating their capacities to reduce superoxide radicals in the reaction. The results were correlated with the findings of Sriram et al. (2016), who reported that the ethanolic extract exhibited a maximum of 88.1% at a concentration of 800 µg/ml among various leaf extracts of T.cordifolia assessed by superoxide radical scavenging activity. Mir Zahoor et al. (2016), reported that the ethanol leaf extract of C. pareira exhibited a maximum of 93.64% at a concentration of 400µg/ml and the IC50 values of ethanol extract showed 55.05µg/ml when compared to the standard ascorbic acid (32.98 µg/ml) which were in agreement with the present study. Such significant scavenging activity may be due to the presence of natural antioxidants such as polyphenols in C. pareira leaves (Castro and Freeman, 2001).
The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir et al., 1995). The presence of reductants such as antioxidant substances causes the reduction of Fe\(^{3+}\)/Ferric cyanide complex to Fe\(^{2+}\)/ferrous form. Therefore, the reducing power of the sample could be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Manian et al., 2008). Samples with higher reducing power have better abilities to donate electrons. Free radicals form stable substances by accepting the donated electrons, resulting in the termination of radical chain reactions. It has been widely accepted that the higher the absorbance at 700 nm, the greater reducing power. In the present study, the ethyl acetate extract showed the highest reducing capacity value (1.52 ± 0.01) at 100 µg/ml concentration and the positive control ascorbic acid had a high reducing capacity of 1.86 ± 0.02 at 100 µg/ml concentration and low reducing activity of 0.85 ± 0.01 at 20 µg/ml concentration. In agreement with the present results, Kolli et al. (2015), who reported that the reducing power of ethyl acetate leaf extract of Morinda tinctoria showed that the extract was potent and the power of the extract was increased with the quantity of sample. The absorbance of the extract at 100 µg/ml was found to be high (0.611) when compared to other extracts. The reducing power of the extracts may serve as a significant indicator of its potential antioxidant activity (Wong et al., 2006). In this assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of test specimen. The presence of reductones have been shown to impart antioxidant action by breaking the free radical chain by donating a hydrogen atom.

The total phenol contents are closely related with antioxidant activity. In the present study, the results showed significant good linear positive correlation...
\( R^2 = 1.000^{**} \) between the total phenol content and \( \text{H}_2\text{O}_2 \) free radical scavenging assay. However, there was no significant correlation between total flavonoid content and antioxidant activity which indicated that there is satisfactorily good relationship between phenolic content and antioxidant activity in \( C. \text{pareira} \) leaf extracts. The strong correlation between the total phenol content and \( \text{H}_2\text{O}_2 \) free radical scavenging activity values in the present study implies that antioxidants in \( C. \text{pareira} \) possess free radical scavenging activity. In consistent with the present study, Zulkefli et al. (2013), who reported that the high antioxidant activity in \( \text{Tinospora crispa} \) extract was due to its high phenol content and there are significant linear positive correlation \( (R^2 = 0.621) \) between the total phenolic content and DPPH free radical scavenging assay in the crude extract. In consonance with the present results, Upadhyay et al. (2014) also reported that the ethanolic bark extract of \( \text{Tinospora cordifolia} \) showed the highest free radical scavenging activity \((71.49\%)\) and the extract also had the highest phenolic content of \( 84.62 \pm 0.12 \text{ mg/g} \). These results suggested that the bark has better antioxidant activity and also better extracted with ethanol and the antioxidant activity \( (R^2 = 0.99) \) was positively correlated with the total phenolic content \( (R^2 = 0.98) \). In conclusion, the present study provides the evidence that the ethyl acetate and ethanol leaf extract of \( C. \text{pareira} \) contains flavonoid and phenolic contents showed potential antioxidant and free radical scavenging activity. These \textit{in vitro} assays demonstrate that this plant is an important source of natural antioxidant which might be preventive against oxidative stresses.

The traditional methodology of studying natural products includes the fractionation of a complex mixture, separation and isolation of the individual components using liquid chromatography and structure elucidation using various
spectroscopic methods (Still et al., 1978 and Sasidharan et al., 2011). A variety of techniques such as chromatography and spectroscopic analysis can be used to determine and estimate the presence of phytoconstituents in medicinal plants. In the present investigation, the ethyl acetate leaf extract exhibited higher activity in antimicrobial and antioxidant assay, hence it was selected for the isolation of bioactive compound by column chromatography. In column chromatography, the resulting forty eight fractions eluted were concentrated using rotary evaporator and an aliquot of all the concentrated fractions were loaded in activated silica gel thin layer chromatography plates with suitable mobile phase, Toluene: Ethyl acetate (5:5) and the spots were visualized by exposing the plates to iodine vapour and UV light. Based on the TLC profile, the fractions having same Rf value were pooled out and combined to give fifteen fractions and numbered as fraction 1 to 15. All the fifteen fractions were screened for in vitro antioxidant activity. Among the fifteen fractions, fraction 13 obtained from first step column chromatography exhibited significant dose dependent inhibition of DPPH activity with the percentage of activity 76.57 compared with other fractions and H2O2 scavenging activity with the percentage of inhibition 63.92 when compared with other fractions. The bioactive fraction 13, which showed highest antioxidant activity was selected for further purification using silica gel. About ten fractions measuring 10ml each were eluted and concentrated by rotary evaporator and an aliquot of all the concentrated fractions were loaded in activated silica gel thin layer chromatography plates and the spots were visualized. Based on the TLC profile, the fractions having same Rf value were pooled out and combined to give seven fractions and numbered as fraction 1 to 7. All the seven fractions were screened for in vitro antioxidant activity. The results clearly demonstrated that among
the seven fractions tested, fraction 4 obtained from second step column chromatography exhibited significant dose dependent inhibition of DPPH activity with the percentage of activity 67.71 and H$_2$O$_2$ activity with 61.72. The bioactive fraction 4 showed highest antioxidant activity, was selected for further purification. About five fractions were eluted and concentrated by rotary evaporator and an aliquot of all the concentrated fractions were loaded in activated silica gel thin layer chromatography plates and the spots were visualized. Based on the TLC profile, the fractions having same R$_f$ value were pooled out and combined to give three major fractions and numbered as MF1 to MF3 and TLC was carried out for each fraction. Among them, fraction MF3 showed single spot on TLC plate and finally the isolated compound was subjected to spectroscopic studies. In accordance with the present findings, Do Nguyen et al. (2015), who reported the isolation of phytoconstituents from the ethyl acetate leaf extract of *Tridax procumbens* by column chromatography. Eight fractions were collected, concentrated and weighed. TLC was performed for eight fractions after concentrating them in a water bath for identification of single spot using ethyl acetate: methanol (90:10, v/v) as solvent system. Fraction (Fr.5) and (Fr.8) showed single spot in TLC, confirming the presence of single compound in the fractions. The isolated bioactive compound eluted from column chromatography was subjected to NMR and Mass spectrometry.

In the present study, the isolated bioactive compound eluted from column chromatography was subjected to NMR and FT-IR studies and Mass spectrometry for the identification and structural elucidation of the compound. The $^1$H NMR spectra of the isolated bioactive compound in chloroform (CDCl$_3$) solution showed four resonance signals in the range 7.9 - 6.9 ppm indicating the presence of four aromatic
protons. The peaks are ppm 7.9 (1H, d, $J = 8$ Hz, H-6), 7.5 (1H, t, $J = 8$ Hz, H-4), 7.0 (1H, d, $J = 8$ Hz, H-3), 6.9 (1H, t, $J = 7$ Hz, H-5). Analysis of the NMR spectra also displayed a singlet at ppm 10.1 corresponds to the carboxyl O-H group. Further, a peak was observed at 5.216 ppm due to -OH group. $^{13}$C NMR spectra, provided seven resonance signals. The resonance at 174.5 ppm was identified to be the chemical shift of carbonyl carbon. The rest of the peaks appeared at 162.2, 136.9, 130.9, 119.6, 117.8, 111.3 ppm corresponds to the chemical shifts of aromatic ring carbons. These spectral features could be attributed to salicylic acid (Mladar Takac et al., 2004).

In order to further confirm the structure, FT-IR spectral studies were performed in the 4000-400 cm$^{-1}$ range. A broad peak around 3238 cm$^{-1}$ was observed in the FT-IR spectra assigned to the stretching frequency of hydroxyl group (O-H). The peaks at 3006, 2856 cm$^{-1}$ corresponds to the aromatic $=$C–H stretching. A strong band at 1657 cm$^{-1}$ in IR spectra was the characteristic of C=O group in the carboxyl part. Since the compound contain an aromatic ring, the region around 1612 cm$^{-1}$ showed absorption band due to C=C carbon skeleton vibration. The other IR peaks are 1483, 1444 (C-H), 1295, 1246 (C–O), 1156 (C-C), 892 (O-H) (in cm$^{-1}$). The mass spectrum of the isolated compound shows a molecule ion peak at M/Z 139 and supports conclusions drawn from the NMR and IR spectrum (Afsar Khan, 2009).

These datas were compared to the spectral data for salicylic acid reported earlier by Banday et al. (2012), who isolated salicylic acid from ethyl acetate - methanol root extract of Conyza canadensis, for the first time. The compound salicylic acid showed melting point 160.5 °C and mass spectra, showed the molecular ion peak at m/z 138.0314. The compound exhibited IR spectra displaying absorptions
at $\nu_{\text{max}} \text{cm}^{-1}$ 3237 due to hydroxyl group, $\nu_{\text{max}} \text{cm}^{-1}$ 1662 for carbonyl group and $\nu_{\text{max}} \text{cm}^{-1}$ 1609 for conjugated C=C, besides other absorption peaks at 2856, 2991, 1440, 1292, 1246, 1154 and 892. The $^1$H-NMR spectrum of compound displayed resonance signals due to aromatic protons at ppm 7.89 (d, $J = 7.3 \text{ Hz}$), 7.48 (t, $J = 7.4 \text{ Hz}$), 7.1 (d, $J = 8.1 \text{ Hz}$) and 6.96 (t, $J = 7.3 \text{ Hz}$), besides a prominent signal at ppm 6.1 (1H, s, COOH). In $^{13}$C-NMR spectrum, compound displayed a prominent resonance signal at $\delta$ C 174.4 for carbonyl carbon and a resonance signal at ppm C 161.8 for a quaternary carbon (C-2). Besides, the $^{13}$C-NMR of the compound showed resonance signals at $\delta$ C 117.3, 119.1, 130.5 and 136.3.

The *in vitro* anticancer and cytotoxicity evaluation was performed in the isolated bioactive compound (Salicylic acid) to investigate its effects against human epithelial ovarian cancer cells. PA-1 and OWA-42 were used as cancer cell lines and L929 fibroblast cells was used as normal cell line (control). The result inferred that, the isolated compound inhibited the growth of PA-1 and OWA-42 cancer cell lines in dose dependant manner and displayed strong cytotoxic activity with lower IC$_{50}$ value 10.22 $\mu$g/ml, when compared to the standard drug doxorubicin with IC$_{50}$ value 5.87 $\mu$g/ml on PA-1 cells and with lower IC$_{50}$ value 11.83$\mu$g/ ml on OWA-42 cells when compared to the standard drug doxorubicin with IC$_{50}$ value 5.32 $\mu$g/ml. According to Kosanic *et al.* (2012), compounds with lower IC$_{50}$ value often indicated higher cytotoxic activity. In the present investigation, it was noticed that PA-1 and OWA-42 treated with higher concentrations of isolated compound had showed various morphological changes such as cell shrinkage, membrane blebbing, loss of integrity and cell adhesion properties which proved that the isolated compound had induced apoptosis in PA-1 and OWA-42 cells and resulted in significant reduction of
cells. It further inferred that the isolated compound had potent cytotoxicity. On the other hand, L929 fibroblast cells treated with isolated compound recorded moderately higher IC$_{50}$ value 35.29 µg/ml, when compared to the standard drug doxorubicin with higher IC$_{50}$ value 61.55 µg/ml. From the present results, it was evident that a maximum concentration of isolated compound had showed 84.55±0.46% growth inhibition, thus indicated the non-toxic nature of the isolated compound towards L929 fibroblast cells. In accordance to the present findings, Noble et al. (1997), who reported the comparative cytotoxicity of 3, 4, and 5-aminosalicylic acid, acetylsalicylic acid (AcSA), and the parent compound salicylic acid (SA) investigated for the free acids and for their sodium salts on four established cell lines such as MDCK, LLC-PK1, NRK (renal cells) and HepG2 (hepatic cells). The free acid compounds were less toxic than their corresponding salts. Chandrappa et al. (2014) reported the in vitro anticancer activity of Quercetin isolated from ethanol extract of Carmona retusa (Vahl.) Masam on Hep G2 cell lines by MTT assay, showed significant cell apoptosis with IC$_{50}$ values 53.07µg/ml and 54.31µg/ml for 24 and 48 hours of incubation. The present study was also corroborated by the findings of Kurata et al. (2007), who isolated dicaffeoylquinic acids (diCQA) from sweet potato leaf and investigated the suppression of the proliferation in human colon cancer cell lines (DLD-1). Surapong Rattana et al. (2016) reported the in vitro anticancer activity of Oxonanolobine isolated from the methanol extract of Tiliacora triandra, possessed moderate activity against lung cancer cell line (NCI-H187) with IC$_{50}$ values of 27.6 ± 4.30 µg/ml when compared to the standard anticancer drugs, doxorubicin with IC$_{50}$ value 0.06 ± 0.00 µg/ml and ellipticine with IC$_{50}$ value 1.16 ± 0.10 µg/ml.
Several anti-cancer drugs are known to show their effects by blocking cell cycle (Stewart and Kleihues 2003). Anti-cancer drugs with minimal side effects on normal cells are highly desirable for therapeutic purposes (Buolamwini 1999). Hence, the current cytotoxicity study against, L929 fibroblast cells revealed that the isolated compound (Salicylic acid) possesses less cytotoxic activity against non-cancerous cells. Agents that are capable of inducing selective apoptosis of cancer cells, without causing much harm to normal cells, have received considerable interest in the development of novel cancer chemotherapeutic drugs (Cotter, 2009). In contrast, doxorubicin, a conventionally used anticancer drug, was found to have cytotoxic and apoptotic effects in cancer cells at the same concentrations. The results suggested that the isolated compound possesses anti-cancer activity in human epithelial ovarian cancer cells, PA-1 and OWA-42, with less cytotoxic effects against normal cells. Thus, it is clearly evident that the isolated compound (Salicylic acid) from ethyl acetate leaf extract of *Cissampelos pareira* var.hirsuta has potential anticancer properties.