DISCUSSION

Herbal medicines have become an integral part of standard healthcare, based on a combination of time honored traditional usage and ongoing scientific research. Burgeoning interest in medicinal herbs has increased scientific scrutiny of their therapeutic potential and safety. Some of the medicinal plants are believed to enhance the natural resistance of the body to infections (Atal et al., 1986). The modulation of immune response with the aid of various bioactives in order to alleviate certain diseases is an active area of interest. Apart from being specifically stimulatory or suppressive, certain agents normalize or modulate pathophysiological processes and are hence called 'immunomodulatory agents' (Wagner, 1983).

Immunomodulatory activity and antioxidant activity of three selected plants i.e. *Sphaeranthus indicus, Cissampelos pareira and Curculigo orchioides* were carried out in order to justify the traditional claims endowed upon them. The bioactive extracts and / or fractions from the plants were identified and established their identity using HPTLC analysis. Isolation of the phytoconstituents has also been carried out to identify the active moieties present in order to develop chemical and /or biological marker for the standardization of the plant.

The studies have been carried out in a systematic manner in order to standardize selected drugs and establish a useful piece of data.

The selected drugs were identifies carefully. Certain diagnostic values were determined for individual drugs which could be used for identification purpose.

The values like ash value, water soluble extractives and alcohol soluble extractives were obtained to set up certain standards in order to avoid batch to batch variation. The data of the analysis suggested, all the three drugs were rich in water soluble extractives. Preliminary phytochemical screening helped in designing methods for preparation of the extracts for the biological screening.

Since the selected drugs were reported to contain phytoconstituents with a variation in their chemical nature, the methods selected for the preparation of the extracts were different in nature.

In case of *S. indicus* methanol extract and its petroleum ether fraction, benzene, chloroform and remaining methanol fraction were selected based on the nature of components like sesquiterpene lactone, flavonoids present. However, water extract was also used in its two fractions, n-butanol and remaining aqueous fraction for biological screening.

In case of *C. pareira*, since alkaloids are reported and which being physiologically active components the extraction was aimed to have alkaloidal fraction from the total methanolic extract and these were subjected to biological activity.

C. orchioides being a rhizome contains mainly polar components was extracted with methanol and methanolic extract was subjected for fractionation with non polar solvents which yielded insignificant quantities of components and therefore for biological activity methanol extract was selected.

The evaluation of immune activity was undertaken using different methods for assessing the immune functions in different manner. Selections of doses for the biological activities were derived from the reported toxicity profiles of the selected drugs.

The effect of selected plants on humoral response was tested by their influence on sheep erythrocyte- specific haemagglutination titre in mice. Methanol extract of *S. indicus* and its petroleum ether, benzene and chloroform fractions and remaining methanol fraction elevated the production of circulating antibody titre. Petroleum ether extract of this plant also found effective in increasing HA titre. However, water extract and its fractions were found to have no effect on the HA titre levels. This indicates the enhanced responsiveness of macrophages and T and B lymphocyte subsets involved in the antibody synthesis.

Methanol extract of *C. pareira* increased the value of HA titre, however, statistically insignificant. The alkaloidal fraction prepared from methanol extract of *C. pareira* showed suppressive effect on antibody production. It was an interesting observation that the total methanol extract has increased the humoral response though not significantly; its alkaloidal fraction suppressed the antibody production. It was already

reported in case of *Withania somnifera* (Agrawal and Singh, 1999), that its alcoholic extract has stimulating effect on production of antibodies, whereas withanolides isolated from it showed suppressive effects.

Methanol extract of *C. orchioides* also increased the humoral response to SRBCs.

Thus, it can be concluded that stimulating effects on humoral immunity by petroleum ether extract, methanol extract of *S. indicus* and its different fractions, methanol extract of *C. pareira* and methanol extract of *C. orchioides* may be due to increased activity of macrophages and T and B lymphocyte subsets. However, the alkaloidal fraction prepared from methanol extract of *C. pareira* has immunosuppressive activity on humoral arm may be due to reversal of the above mechanism.

The enhanced uptake of particulate matter with the treatment of petroleum ether extract, methanol extract and its different fractions, water extract and its n-butanol fraction of *S. indicus*, methanol extract of *C. pareira* and methanol extract of *C. orchioides* was evident from carbon clearance test. Increased activities of phagocytes may enhance antigen processing and presentation and in turn, at least in part, augment the antibody responses [Mungantiwar et al., 1999]. Similar effects were observed with the extracts studies for the activities.

The results of the studies indicated that the increase in carbon clearance index by *S. indicus, C. pareira* and *C. orchioides* may be due to the enhancement of phagocytic function of mononuclear macrophage and non- specific immunity.

In case of *S. indicus* it was noted that methanol extract and its different fractions stimulated the DTH response as evidenced by increased paw thickness in mice. Water extract and its fractions showed no response on DTH. Methanol extract of *C. pareira* and *C. orchioides* also had stimulatory effect on the DTH reaction. However, alkaloidal fraction of *C. pareira* showed suppressive effect on DTH.

The above observations indicated that increase in the DTH response by *S. indicus, C. pareira* and *C. orchioides* may be due to stimulatory effect on lymphocytes and necessary cell types required for the expression reaction.

Since drugs augmented the circulating antibody titre, it was thought worthwhile to evaluate their effect cyclophosphamide-induced myelosuppression. A high degree of cell proliferation renders the bone marrow a sensitive target particularly to cytotoxic drugs. In fact bone marrow is the organ most affected during any immunosuppression therapy with this class of drugs. Loss of stem cells and inability of the bone marrow to regenerate new blood cells results in thrombocytopenia and leucopenia (Agarwal et al., 1999). In the case of cyclophosphamide induced myelosuppression, remaining methanol fraction of methanol extract was able to bring back the levels of WBC to the normal while other fractions were ineffective suggesting that polar components responsible for protection against cyclophosphamide myelosuppression. Water extract and its different fractions were also failed to offer any protective effect against cyclophosphamide. Administration of the methanol extract of rhizomes of *C. orchioides* and roots of *C. pareira* was found to increase the total WBC count which was lowered by cyclophosphamide, a cytotoxic drug. The protective effect offered by these drugs against myelosuppression induced by cyclophosphamide was comparable with those obtained for *Asparagus racemosus* and *Withania somnifera* (Thatte and Dahanukar, 1988; Davis and Kuttan, 1998).

Hence, it was established from the above study that *S. indicus, C. pareira* and *C. orchioides* possessed ability to counteract the myelosuppressive effects of the cytotoxic drug, cyclophosphamide by stimulating the bone marrow activity.

Statistically significant rise in HA titre, DTH response and phagocytic activity by petroleum ether and remaining methanol fraction of *S. indicus* suggest that active principles of flower heads which are responsible for stimulation of antibody response, cell mediated response and phagocytic function can be extracted with both polar and non polar solvents.

Qualitative chemical tests showed presence of mainly phenolics, steroids and terpenoids. The sesquiterpenes are widely distributed in plant flora particularly in *Compositae*. The pharmacological activity of the

sesquiterpenes ranges from neurotoxic to antimicrobial. Santonin, a sesquiterpene lactone from *Artemisia maritima* (wormwood) was used in the medicine for the elimination of intestinal worms; similarly derivatives of Chinese medicine quinghaosu, artemisinin, obtained from *Artemisia annua*, have recently been recommended for the treatment of resistant strain of malaria. This compound contains unusual peroxide which is associated with its biological activity (Singh, 2002). The sesquiterpene lactones, isolated from the *Tridax procubens* were known to cause delayed type hypersensitivity (Picman, 1986). Sesquiterpene lactones from *Radix inulae* were found to possess significant anti-inflammatory and hepatoprotective activities similar to that of silymarin, and antidematophytic and antifungal activities (Wenhua, et al., 2004). Triterpenoides and sesquiterpenes were reported responsible for the formation of nanoconstructs largely referred to as immunostimulatory complexes (ISCOMS). These ISCOMS may presumably be held responsible for stimulation and potentiating of overall immune response (Picman, 1986).

Recently three new Sesquiterpene glycosides isolated from *Dendrobium nobile* were reported for their immunomodulatory potential (Ye, et al., 2002). During the present study Sesquiterpene lactones were found in non-polar fractions while remaining methanol fraction was devoid of these constituents. The activity of petroleum ether, benzene and chloroform fractions may therefore be attributed to the presence of such non-polar type of compounds. However, protection against cyclophosphamide myelosuppression was observed by only remaining methanol fraction, suggests the content of constituents other than sesquiterpenoids which were also responsible for increased humoral and cellular response.

Sterols are derivatives of steroid. Modern clinical studies have supported their role as anti-inflammatory and analgesic agents. â-sitosterol has significant hypolipidemic activity (Singh, 2002).

Some studies have already established the immunomodulatory activity of non polar constituents of the plants. Lipid part isolated from seeds of *Nyctanthes arbortristis* was found responsible for increased haemagglutination antibody titre (Puri, et al., 1994). Sesquiterpene lactone fraction obtained from *Sassurea lappa* showed anti-inflammatory activity, that may be the reason petroleum ether extract at high doses reduced the DTH response (Damre, et al., 2003).

C.pareira contains bisbenzoylisoquinoline alkaloids, possessing the anti-inflammatory potential as shown in clinical experiments. These alkaloids suppressed the production of nitric oxide, a critical mediator in inflammation, which explain some aspects of the anti-inflammatory mechanisms present in the alkaloids of *C.pareira* (Kondo et.al., 1993). The present study also found in accordance with this. Alkaloidal fraction of *C. pareira* suppressed DTH response in dose dependent manner, it supports previous findings. Recently, many isoquinoline alkaloids are under intensive study for their antimicrobial, anti-tumor, neuropharmacological and immunosuppressive action. Some of them are already in clinical use and it is of interest to investigate their possible effect on the immune system (Ivanovska, 1997).

Some of the constituents reported from *C. orchioides* include triterpenoids (Mehta and Gawarikar, 1991), saponins (Kubo et.al., 1983; Xu, et. al., 1992b) and phenolics glycosides (Garg et. al., 1989). Saponins, in particular, are described as immunostimulatory agents (Liu, et al., 1995). Recently phenolic glucosides isolated from *C. orchioides* were found to be responsible for the enhancement of HA titre and DTH response (Lakshmi et al., 2003).

The results of screening of immunomodulatory activity helped in identifying the potent bioactive extracts and /or fractions of the extract from the selected drugs. Increased humoral response as well as cellular mediated immunity and protection offered against cyclophosphamide prompted us to undertake the immunomodulatory activity of bioactive extracts in cyclophosphamide induced immunosuppressed animals.

The protective effect of residual methanol fraction (bioactive fraction) of *S. indicus*, methanol extract of *C. orchioides* and methanol extract of *C. pareira* against cyclophosphamide-induced immunosuppression in mice was assessed in the present investigation. The results obtained showed that residual methanol fraction of *S. indicus*, methanol extract of *C. orchioides* and methanol extract of *C. pareira* displays a dose dependent immunostimulatory effects in relation to antigenic stimulation.

In the same theme many natural extracts showed immunostimulating activities in immunocompromised animals. Makare et al. (2001) reported reversal of suppression of antibody titre by cyclophosphamide in mice with the treatment of ethanol extract of *Mangifera indica* containing 2.6% mangiferin in dose dependent manner. Hafeez et al. (2001) also reported protective effect of total aqueous extract of *Cassia occidentalis* against cyclophosphamide induced immunosuppression of humoral immunity in mice. Botanical based immunomodulators are often employed as supportive or adjuvant therapy to overcome the undesired effects of cytotoxic chemotherapeutic agents and to restore health to normal. Diwanay et al. (2004) had reported various immunopharmacological activities of *Withania somnifera*, *Tinospora cordifolia*, *Asparagus racemosus* in cyclophosphamide treated mouse ascitic sarcoma. Simultaneous treatment with different extracts of these plants resulted in protection towards cyclophosphamide induced immunosuppression as evident by significant increase in white cell counts and haemagglutinating antibody titre.

It was noted in case of all the three selected drugs, in both the pretreatment schedules 200 and 400 mg/kg doses were found most effective in inducing immune functions. It appears that range of 200-400 mg/kg is the optimum dose in mice. An increase in dose might have induced down regulation of immune functions. The response at higher dose was either identical to control or stimulated as compared to control animals. The exertion of an immune response needs a certain threshold dose, beyond which it does not show any enhancement, and it has also

been reported that immunostimulating substances could exhibit suppressive effects (Wagner and Jurcic, 1991).

Anti oxidizing agents are those essential compounds that improve the body physiological self-defense against cancer, cardiovascular and neurodegenerative diseases, HIV, etc. [Cren et al., 1999]. Several antioxidants have been reported to possess immunomodulatory properties [La Fuente and Victor, 2000; Ruby et al., 1995; Devasagayam and Sainis, 2002]. Therefore it was thought worthwhile to undertake antioxidant activity of the bioactive extracts and /or fractions of the selected plants, to establish their immunomodulatory effect through antioxidant activity.

The *in vitro* free radical scavenging activity of these drugs was assessed in terms of their ability to bleach the stable radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH). DPPH is a stable free radical in aqueous or ethanol solution and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997). In order to evaluate antioxidant potency through free radical scavenging with the test samples, the change in the optical density of DPPH radicals is monitored. Hence, DPPH· is usually used as a substrate to evaluate antioxidative activity of antioxidants (Duh et al., 1999). Methanol extract and its residual methanol fraction of *S. indicus*, methanol extract and alkaloidal fraction of *C. pareira* and methanol extract of *C. orchioides* exhibited scavenging activity of DPPH. Methanol fraction of *S. indicus*, alkaloidal fraction of *C. pareira* and methanol extract of *C. orchioides* were strong scavengers of DPPH. Whereas, other tested extract and /or fractions of these plants hold only moderate activity.

Superoxide radical is known to be very harmful to cellular components as precursor of more reactive oxygen species (Halliwell and Gutteridge, 1985). The NBT (Nitro blue tetrazolium) reduction method showed that the methanolic extracts of *S. indicus*, and *C. pareira* possessed moderate, while bioactive fraction of *S. indicus*, alkaloidal fraction of *C. pareira* and methanol extract of *C. orchioides* were strong

scavengers of superoxide radical generated in riboflavin-NBT-light system *in vitro* as compared to the standard, ascorbic acid.

The measurement of reductive ability was done by Fe3+-Fe2+ transformation in the presence of samples and standard antioxidant, ascorbic acid (Oyaizu 1986). The reducing power is associated with antioxidant activity (Duh et al., 1999). Methanolic extracts of *S. indicus*, and *C. pareira*, and *C. orchioides* possessed moderate reducing power, while bioactive fraction of *S. indicus* and alkaloidal fraction of *C. pareira* exhibited activity comparable to standard, ascorbic acid.

In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Marletta, 1989). Methanol extract of *C. orchioides* only showed moderate activity in scavenging nitric oxide compared to standard Curcumin All the other extracts and /or fractions from *S. indicus* and *C. pareira* failed to show any scavenging effect against nitric oxide radical.

Lipid peroxidation is initiated by radicals attacking unsaturated fatty acids, and propagated by a chain reaction cycle (Shimazaki, 1994). Since unsaturated fatty acids are most important components of biological membranes and impart desirable properties upon the fluidity of cellular membrane structure, the peroxidation of unsaturated fatty acids in biological membranes leads to disruption of membrane structure and function (Machlin and Bendich, 1987). In particular O^{-}_{2} and ·OH induce various injuries to the surrounding organs and play a vital role in some clinical disorders. Therefore removal of O^{-}_{2} and ·OH is the most effective defense of the living body against disease (Lin et al., 1995). Any compound – natural or synthetic – with antioxidant properties might totally or partially alleviate this damage. Inhibition of the lipid peroxidation induced by iron/ADP/ascorbate complex was noted with the extracts studied. However, strong activity was noted with bioactive fraction of *S. indicus*, methanol extract of *C. orchioides* and alkaloidal fraction of *C. pareira*. Methanol extract of *S. indicus* and *C. pareira* hold the moderate activity.

Recent studies showed that a number of plants products include polyphenolic substances such as flavonoids and tannins. Those natural

antioxidative substances usually have a phenolic moiety in their molecular structure. They have been found among flavonoids, tocopherols and catechines. Organic acids, carotenoids, protein hydrolysates and tannins can act as antioxidants or have synergistic effects when used together with phenolic antioxidants. Phenolic antioxidants are potent free radical terminators (Shahidi and Wanasundara, 1992). Phenolic compounds, biologically active components, are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step. This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups (Sawa et al., 1999).

In present study all the three plants revealed the presence of phenolic compounds and one can therefore substantiate their activity pertaining to the chemical composition. Tetrandrine (Rojanasonthorn, 1970) and berberine (Anwar et.al., 1968) were previously isolated from *C.pareira* roots. They were also reported for their antioxidant activity in various models (Cao, 1996 and Hwang, et.al., 2002). Activity of this drug may be due to presence of such kind of alkaloids with other alkaloids. Rhizomes of *C. orchioides* found to contain phenolic glucosides (Kubo et al., 1993), such type of compounds may be responsible for the activity of this drug.

Thus from the present study it can be concluded that *S. indicus*, *C. pareira* and *C. orchioides* possess different levels of antioxidant activity and this antioxidant activity may be one of the mechanism by which these drugs are exerting immunomodulatory activity.

In the past few years high performance thin layer chromatography has emerged as a potential tool for rapid and useful phytochemical evaluation of herbal drugs (Indian Herbal Pharmacopoeia, 1998; Houghton, 1999). HPTLC fingerprint profiles established for bioactive extracts and /or fractions to identify them.

Bioactive extracts based on the observations were selected and subjected to isolations of the phytoconstituents. Some of the compounds were isolated from petroleum ether extract of *S. indicus*, petroleum ether

fraction of methanol extract of *S. indicus,* residual methanol fraction of methanol extract of *S. indicus,* methanol extract of *C. pareira* and methanol extract of *C. orchioides.* Isolated compounds were subjected to different spectral studies in order to derive their structure.

Characterization of compounds showed that compounds isolated from *S. indicus* exhibited aliphatic nature. Compounds S1 to S8 showed important functional group indicating their aliphatic nature. Compounds isolated from petroleum ether extract and petroleum ether fraction of methanol extract, S3 and S7 showed characteristic bands in IR spectra at 1746 and 1705, indicating presence of ester and ketone carbonyl groups. These compounds also showed characteristic absorption in the region 720- 734 for long chain of methyl groups. Thus, it can be said that isolated compounds were either aliphatic long chain alcohols or esters or ketones.

Compound C1 isolated from methanol extract of *C. pareira* showed characteristic absorption in the region 1740 in IR spectra, indicating ketonic group. NMR spectra suggested its aromatic nature. Compound C2 was alkaloid and elemental analysis suggested the skeleton $C_{14}H_{23}NO_8$, however, due to inaccuracies in the mass spectra exact molecular formula couldn't derive. NMR suggested tri substituted structure.

The data for compound isolated from *C. orchioides* also suggested it to be aliphatic long chain alcohol.

The exact structures of the compounds could not be elucidated due to lacking of spectral details and confirmatory chemical tests. However, the data available provided preliminary information regarding these compounds.