TISSUE CULTURE STUDIES

Isodon is a cosmopolitan and important genus of the family Lamiaceae. Isodon wightii (Syn: Plectranthus wightii) is distributed in Western Ghats of South India. A survey of literature did not reveal any reference to a previous work on in vitro propagation of I.wightii. The present study on I.wightii was undertaken with the aim to standardize an protocol for mass multiplication using various explants, to isolate and analyze the secondary metabolite and to test the antibacterial, cytotoxicity, antioxidant activity and anticarcinogenic activity of the isolated compound.

In I.wightii the conventional method of propagation is by seeds. However, the conventional propagation method is beset with the problems of poor seed germination. In vitro culture techniques offer a viable tool for mass multiplication and germplasm preservation of rare, endangered, aromatic and medicinal plants (Kumar and Seeni, 1998; Faisal et al., 2006 and Siddique and Anis, 2007).

The success of in vitro culture is largely dependent on three factors, explant choice, media composition and control of physical environment (Thorpe and Patel, 1984). The important of the choice of the explant, which serves as the inoculum for axenic culture, is well documented in the classical compilation of George and Sherrington (1984). In vitro culture and in particular, nodal cultures have been successfully used to conserve threatened and medicinal plants (Tideman and Hawkers, 1982; Sharma and Chandel, 1992; Ramulu et al., 1999 and Beena et al., 2003). In a majority of the members of Lamiaceae, nodal and leaf explants have been employed successfully for regeneration. In the present study also, nodal and leaf explants responded favourably for regeneration.

Murashige and Skoog (MS) medium was found to be frequently employed for in vitro culture in Lamiaceae (Ahuja et al., 1982; Carlos and Carmen, 1996; Savithri Bhatt et al., 2001; Rani et al., 2006; Makunga and Van Staden, 2008). LS medium was found to be used in Lavandula dentata (Sudria et al., 1999) and B5 medium in Salvia canariensis (Mederos Molina, 2004). In accordance with above observations, half strength MS medium showed maximum number of responding cultures from the node and leaf explants of I.wightii.
The requirements and nature of exogenous hormones depend on the endogenous level in the plant system, which is variable with plants, type of tissues and physiological state of the explants. A survey of literature pertaining to the multiple shoot induction from the nodal and leaf explants in Lamiaceae revealed that addition of exogenous cytokinins is necessary before successful regeneration can occur.

In *I. wightii* the nodal explants failed to respond morphogenetically to a growth regulator-free MS medium. BA at all concentration tested was more effective in shoot induction as compared to TDZ, Kin and Zea. BA at 1 mg/l was found to be the optimum concentration for inducing maximum number of shoots (6.00) followed by TDZ, Kin and Zea. These results are consistent with earlier findings in *Ocimum americanum* and *O. canum* (Sitakanta Pattnaik and Pradeep Chand, 1996) and *O. basilicum* (Sahoo et al., 1997; Begum et al., 2002 and Siddique and Anis, 2008a) where BA was most effective than other cytokinins in inducing shoot buds. The percentage of bud break and multiple shoot induction declined with the increasing concentration of all cytokinins than the optimum level. Moreover, the shoots which developed at higher concentration failed to elongate. Lowering the concentration of cytokinins in micropropagation studies to achieve higher rate of multiplication has also been reported in *O. basilicum* (Sahoo et al., 1997) and *Holarrhena antidysenterica* (Kumar et al. 2005).

Enhancement of shoot elongation was achieved by subculturing the shoots in half strength MS medium supplemented with each cytokinin in combination with GA₃ (0.5mg/l). Among all the cytokinins and GA₃ combination tested, the maximum number of shoots (20.25) and shoot length were obtained on half strength MS medium supplemented with BA (1mg/l) and GA₃ (0.5 mg/l). Similar results were obtained in *Ocimum basilicum* (Sahoo et al., 1997), *Salvia sclarea* (Liu et al., 2000) and *S. broussonetii* (Mederos Molina, 2006) where BA in combination with GA₃ resulted in marked increase in multiple shoot formation and elongation. The positive effect of the combination of BA with GA₃ on shoot proliferation has also been reported in *Prunus americana* (Georgios Koubouris and Miltiadis Vasilakakis, 2006). In contrast, IAA promoted proliferation and elongation of shoots in *Ocimum basilicum* (Siddique and Anis, 2007 and 2008a), *O. gratissimum* (Ahuja et al., 1982), *Salvia nemorosa* (Ewa Skala
and Halina Wysokinska, 2004), *Lavandula vera* (Quazi, 1980), *L.latifolia* (Calvo and Segura, 1989). NAA promoted proliferation and elongation of shoots in *Petasites hybridus* (Wldi et al., 1998), *Eucalyptus grandis* (Luis et al., 1999) and *Hybanthus enneaspermus* (Prakash et al., 1999). It is apparent that in *Isodon wightii*, the initial stage of bud break require the presence of BA (1mg/l) but their further growth proliferation and elongation demanded to transfer a medium containing BA (1 mg/l) and lower concentration of GA$_3$ (0.5 mg/l). The statistical analyses reveal significant effect of culture media, cytokinin concentration, number of shoot and shoot length (Table-3).

Shoot organogenesis from callus cultures can be used as an effective method for multiplication medicinal plants (Sarasan et al., 1994 and Lusia and Rojas, 1996). In the present study callus mediated shoot organogenesis was chosen as an alternative method to achieve a higher rate of shoot multiplication.

The influence of growth regulator on callus development from leaf explant was investigated using various auxins. Among the auxins tested, 2, 4-D induced profuse callusing from the leaf explant. However, the frequency of callus induction was low. A combination of NAA (1 mg/l) + BA (0.5 mg/l) was observed to be the best for profuse callus induction. These results are in agreement with Dronne et al. (1999) on *Lavandin* where callus induction was obtained when both auxin and cytokinin were added to the culture media. The combined effect of cytokinin and auxin on callus formation was also reported in *Lavandula latifolia* (Calvo and Segura, 1988) and *Salvia officinalis* (Kintzios et al., 1999).

Most of the earlier reports pertaining to shoot regeneration from the callus in members of the family Lamiaceae have suggested the effective role of BA in shoot initiation, either individually or in combination with other growth regulators (Misra, 1996; Sen and Sharma, 1991 and Sunnichan and Shivanna, 1998). In *Isodon wightii* BA in combination with NAA failed to induce shoot buds. For further amplification and continuous induction of shoot buds, the leaf derived callus of *I.wightii* was subcultured in half strength MS medium fortified with BA and GA$_3$. BA at 1 mg/l and GA$_3$ at 1mg/l favoured shoot regeneration. A similar effect of the both hormones was reported by
Mederos Molina (2006) in Salvia broussonetii. According to Arora and Bhojwani (1989), BA + GA<sub>3</sub> combination markedly enhanced the frequency of multiple shoot formation in Saussurea lappa. GA<sub>3</sub> is known to have stimulatory effect on stem elongation in higher plants (Phinney, 1984). In contrast MS medium supplemented with IAA and NAA produced highest frequency of regeneration and highest number of shoots and longest shoot from the leaf derived callus of Coleus forskohlii (Sairam Reddy et al., 2001) and NAA + BA + GA<sub>3</sub> in Salvia sclarea (Liu et al., 2000). Morimoto et al. (1994) obtained shoots from callus tissue of S.miltiorrhiza on MS medium containing BA and IAA.

A perusal of literature pertaining to the rooting of the microshoots in Lamiaceae revealed that auxins such as IAA, IBA and NAA were used for rooting (Sharma et al., 1991; Mederos Molina et al., 1997 and Ewa Skala and Halina Wysokinska. 2004). Success of auxin free basal medium for efficient root induction was also reported in Salvia miltiorrhiza (Morimoto et al., 1994), S. bancoana, S. valentine (Cuenca and Amo-Marco, 2000) and Coleus forskohlii (Sairam Reddy et al., 2001).

In the present study auxin was needed for the induction of roots. Of the three auxins tested, IBA was the most effective. The maximum frequency of root formation and number of roots was achieved in half strength MS medium supplemented with IBA (1mg/l). Optimum rooting response using IBA was reported for several species including Lavandin (Dronne et al., 1999), Cunila galioides (Fracaro and Echeverrigaray, 2001), Orthosiphon spiralis (Elangomathavan et al., 2003) and Ocimum basilicum (Siddique and Anis, 2007). Both IBA and NAA were effective for rooting in Lavandin and Lavandula stroechas (Panizza and Tognoni, 1991; Nobre, 1996), Ocimum americanum and O. canum (Sitakanta Pattnaik and Pradeep Chand, 1996) and Salvia canariensis (Mederos Molina et al., 1997 and 2004).

Hardening is crucial step prior to transplantation of plant to soil. Different types of potting media like vermiculite, soilrite, sand and peat moss used for the establishment of various Lamiaceae members (Singh and Sehgal, 1999; Elangomathavan et al., 2003; Makunga and Van Staden, 2008; Siddique and Anis, 2008a). The well developed plantlets of Isodon wightii were hardened in various potting media. Plants were well established in
the potting mixture consisting of coir pith, vermiculite and soil in the ratio of 1:1:1 and
the regenerated plants showed 70% survival rate.

**PHYTOCHEMISTRY**

*Isodon* belonging to the Lamiaceae family has attracted considerable attention
because of its various biological activities including antibacterial, antifungal and anti-
inflammatory effects as well as its clinical use in the treatment of human cancer.
Diterpenoids are the major chemical constituent of *Isodon* species. The structures of
many diterpenoids constituents especially those with an *ent*-kaurene skeleton have been
isolated and structure elucidated. Out of 610 known *Isodon* diterpenoids, 212 found in 36
*Isodon* species belong to the C-20 non-oxygenated *ent*-kaurenes, and 190 of them have
been identified since 1984 (Sun *et al.*, 2006).

The closer taxonomic relationships better the chances for the occurrence of similar
compounds in plant taxa. When such compounds are of medicinal or pharmaceutical
importance, attempts are made to search for similar or related compounds. *Isodon* species
being rich in *ent*-kaurene diterpenoids of different oxygenation and cleavage patterns, in
the present study *I. wightii* has been investigated to isolate and elucidate the structure of
the bioactive compound from the leaves.

After repeated column chromatographic purification on silica gel, the acetone
extract of the leaves of *I. wightii* yielded melissoidesin (M). Melissoidesin (M) obtained
as colorless needles and analysis of its $^1$H NMR spectral data revealed 34 protons and $^{13}$C
NMR spectral data revealed the presence of 22 magnetically non-equivalent carbon
atoms. The presence of OH stretching and ester carbonyl stretching were obtained from
the IR spectra confirmed the functional groups. GC-MS shown to possess a molecular
formula of $\text{C}_{22}\text{H}_{34}\text{O}_5$ from the positive molecular ion peak observed at $m/\zeta$ 378.
The compound was also confirmed by its mixed melting point of 220-222° C.

All the spectral and analytical results supported the compound as melissoidesin
(M) (3β, 11β, 15β- trihydroxy -6α-acetoxy-ent- kaur-16-ene) similar to melissoidesin
(M) isolated from the aerial parts of *I. melissoides*. The compound melissoidesin (M) was
identical to melissoidesin F (Zhao et al., 1999a) except for the substituent at C-3. The acetoxyl group at C-3 of melissoidesin F was replaced by a hydroxyl group in melissoidesin M, which was proved by the HMBC correlations of Me-18 and Me-19 (Δ1.12, 1.25, each 3H, s) with C-3 and H-6 (Δ 5.73 1H, br s) with the acetoxy carbonyl carbon, H-11 (Δ 4.30 1H, br s) with C-8 and C-13, and H-15 (Δ 3.98 1H, d, J) 10.0 Hz) with C-9, C-14, and C-16. According to the cross-peaks in the HMBC spectrum of melissoidesin M, the acetoxy group was placed at C-6, and three hydroxy groups were placed at C-3, C-11 and C-15 respectively groups (Zhao et al., 2004).

C-20 non-oxygenated ent-kaurene diterpenoids are largest group of known Isodon diterpenoids and appears to be the most widely distributed. C-20 is always an isolated methyl and C-15 is generally functionalized by a ketone or hydroxyl group. The most representative species that produced C-20 non-oxygenated ent-kaurene diterpenoid is I. angustifolius subsp. glabrescens. From this species, 25 diterpenoids with highly oxygenated structures, glabcensins A-Y and four previous known ones, 7-acetyl-lushanrubescencin A, lushanrubescencins A and B, and rabdoforrestin A were isolated (Zhao et al., 1997 and 1999b).

Melissoidesins, a C-20 non-oxygenated ent-kaurene diterpenoids with different functionalities and diverse groups were reported from I.melissoides. Zhao et al. (2003) reported three new 11β, 16β-epoxy-ent-kauranoids, melissoidesins I-K and one new ent-abietanoid melissoidesin L from the aerial parts of I. melissoides. Other C-20 nonoxygenated ent-kaurene diterpenoids like melissoidesin N-U were also reported in I.melissoides with different functional groups (Zhao et al., 2004). Melissoidesin A-D, V and W were reported from the aerial parts of I.melissoides (Zhao et al., 2005). Melissoidesin G a new diterpenoid purified from I.melissoides was reported by Yu et al. (2007). When compared with other melissoidesins from several Isodon species, the melissoidesin (M) isolated from I.wightii showed the difference in the presence functional groups at the same time all those C-20 non-oxygenated ent-kaurene diterpenes, melissoidesins bear similar oxidation patterns (Sun et al., 2006).
ANTIOXIDANT STUDIES

Extensive studies have been carried out on both well known and less popular medicinal plants of the Lamiaceae family. Some species such as rosemary, sage, thyme, oregano and basil were thoroughly studied for their antioxidant and radical scavenging properties (Couladis et al., 2003; Javanmardi et al., 2003). *Isodon (=Plectranthus)* is a large and wide spread genus with a diversity of ethnobotanical uses. Monoterpenes, sesquiterpenes, diterpenes and phenolics have been reported in species of *Isodon*.

Among these substances, *ent*-kaurenes and abietanes are commonly found in *Isodon* genera have the ability to scavenge free radicals (Guoan Liu et al., 2006; Ahmed Kabouche et al., 2007). In the present study *Isodon wightii* was chosen to screen the antioxidant activity of melissoidesin (M) isolated from the acetone extract of leaves. Melissoidesin (M) was found to show significant scavenging activity on DPPH radicals. Generally phytochemicals recognized as possessing potent antioxidant activity are also strong scavengers of the synthetic nitrogen centered free radical DPPH (Depkevicius et al., 2002). The results of the DPPH free radical scavenging activity suggest that melissoidesin (M) was capable of scavenging free radicals in dose dependent manner and thus may be able to prevent the initiation of free radical mediated chain reactions by stabilizing reactive species. The result was consistent with DPPH radical scavenging activity of abietane diterpenoids such as carnosic acid and carnosol. Orthodihydroxyl groups on aromatic ring C of carnosic acid has been explained by inhibition of the oxidation through donating H atoms to scavenge free radicals produced by DPPH (Miura et al., 2002; Kosar et al., 2004). Like carnosic acid, presence of hydroxyl groups in melissoidesin (M) may be responsible for donating H atoms to scavenge DPPH radicals.

DPPH radical scavenging activity of melissoidesin (M) also confirms the findings of Ahmed Kabouche et al. (2007) where diterpenoids like 7-oxoroleanone-12-methylether, 7α-acetoxyroyleanone-12-methylether, royleanone, horminone, 7-acetylhorminone, cryptojaponol and inuroyleanol isolated from the root of *Salvia barrelieri* showed potent DPPH radical scavenging activity. The abietane diterpenoid hinokiol and the mixture of hexacosan-1, 26-diol and octacosan-1, 28-diol diferulates isolated from *Plectranthus*
*strigosus* also showed potent DPPH radical scavenging activity (Marques *et al.*, 2008). On contrary none of the *ent*-kaurene diterpenoids like enmein, oridonin and nor nodosin isolated from *Isodon japonica* showed DPPH radical scavenging activity (Noriyoshi Masuoka *et al.*, 2006).

Melissoidesin (M) at various concentrations showed potent reducing power activity. The reducing properties are generally associated with the presence of reductones which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Like DPPH radical scavenging activity the reducing power activity of melissoidesin (M) increased with increasing concentration.

The observed reducing power activity of melissoidesin (M) are in agreement with the report of Dorman *et al.* (2004), where the hydrodistilled freeze dried extract of *Satureja cuneifolia* were capable of reducing iron (III), and capable of donating electrons. This property suggests that the extracts may act as free radical chain terminators, transforming reactive free radical species into more stable nonradical products. Ethanolic extract of *Ocimum sanctum* and lyophilized aqueous extract of *Mentha spicata* showed reducing power activity in a concentration dependent manner and the activity was increased with increasing concentration (Juntachote and Berghofer, 2005; Sweetie Kanatt *et al.*, 2007). The same condition was also observed in the reducing power activity of melissoidesin (M). Rosmarinic acid, a phenolic diterpene obtained from different solvent extracts of *Salvia virgata* showed potent reducing power activity. The ability of rosmarinic acid to reduce iron (III) to iron (II) represents its ability to donate electrons (Kosar *et al.*, 2008). Like rosmarinic acid the observed reducing power activity of melissoidesin (M) may be attributed to donate electrons.

Hydroxyl radical is biologically relevant and extremely reactive oxygen species, which can rapidly react and degrade with susceptible food and biologically relevant substrates, such as polyunsaturated fatty acids, proteins, carbohydrates and DNA (Halliwell *et al.*, 1992). Hydroxyl radical scavenging activity of melissoidesin (M) was effective to scavenge the hydroxyl radicals generated via Fenton reaction. There was a correlation between increased concentration of melissoidesin (M) and reduction of hydroxyl radicals.
The observed hydroxyl radical scavenging activity of melissoidesin (M) may be attributed to the presence of electron donating groups. The same condition was also reported in carnosol and carnosic acid isolated from rosemary (Aruoma, et al., 1992). Essential oils such as thymol, γ-terpinene, ρ-cymene, carvacrol, and borneol from Thymus pectinatus (Vardar Unlu et al., 2003) and caffeic acid and its derivatives from Salvia officinalis and S. miltiorrhiza and hydrodistilled extract of Ocimum basilicum were shown to have high reaction rate towards hydroxyl radicals (Bors et al., 2004; Iris Hinneburg et al., 2006). The results of this study were also supported the hydroxyl radical scavenging activity of melissoidesin (M).

The hydroxyl radical scavenging activity of melissoidesin (M) may be related to their hydroxyl groups in its basic structure. However, these properties of putative antioxidant have been attributed to various mechanisms, among which are prevention of radical chain initiation, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging (Diplock, 1997).

Fe$^{2+}$ ion is the most powerful prooxidant among various species of metal ions (Halliwell and Gutteridge, 1984). The ent-kaurene diterpenoid, melissoidesin (M) showed metal chelating activity in dose dependent manner. Initially ferrozine can quantitatively form complexes with Fe$^{2+}$ further the complex was inhibited by melissoidesin (M). In the presence of chelating agents, the complex formation of ferrous and ferrozine is disrupted, resulting in a decrease in red color of the complex. It was reported that chelating agents, which form σ-bonds with a metal are effective as antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Gordon, 1990).

The data obtained from iron chelation revealed that melissoidesin (M) has an effective capacity for metal binding, suggesting that its action as an antioxidant may be related to its iron binding capacity. Similar observations were made with aqueous extracts of Thymbra spicata, Satureja cuneifolia, Coridothybus capitatus, Majorana hortensis, Origanum syriacum, Origanum minutiflorum and Origanum onites (Dorman et al., 2004) and Coleus aromaticus interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine.
(Kumaran and Joel Karunakaran, 2006). Terpenes such as 3β-acetylmonogynol A, 3β-acetyl, 22β-hydroxymonogynol A and 3β-acetyl, 21β, 22β-dihydroxymonogynol A isolated from *Salvia macrochlamys* also supported the metal chelating activity of melissoidesin (M) (Topcu et al., 2007).

Peroxidation of lipids is a principal cause of the oxidative deterioration of susceptible foodstuffs and the loss of physiological function in cellular organelles within the human body (Yamamoto and Niki, 1991). The antioxidant capacity of melissoidesin (M) was the ability to lower the degree of lipid peroxidation induced by hydroxyl radical generated by iron/ascorbate system. Several Lamiaceae species such as, *Origanum majorana*, *Salvia fruticosa* and *Origanum dictamnus* (Triantaphyllou et al., 2001), *Teucrium chamaedrys*, *T. montanum* and *T. polium* (Tatjana Panovska et al., 2005) showed potent antioxidant activity against free radicals formed during linoleic acid oxidation.

The presence of melissoidesin (M) in the linoleic acid emulsion was able to scavenge hydroxyl radicals and minimize the risk of oxidation. The same result was also reported by Ahmed Kabouche et al. (2007) in *Salvia barrelieri* where abietane diterpenoids such as, 7-oxoroyleanone-12-methyl ether, 7α-acetoxyroyleanone-12-methyl ether, royleanone, horminone, 7-acetylhorminone, cryptojaponol and inuroyleanol showed potent antilipid peroxidation activity.

A modified thiobarbituric acid reactive species was used to measure the lipid peroxide formed, using egg-yolk homogenates as lipid rich media. Iron can stimulate lipid peroxidation by Fenton reaction and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxy radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Chang et al., 2002b). The presence of melissoidesin (M) in the lipid peroxidation reaction mixture lead to a reduction of the extent of peroxidation in egg yolk medium and prevented the oxidation of lipid molecules. This inhibition of lipid peroxidation may be either due to chelation of iron or by free radical trapping as suggested by Nidhi Pandey et al. (2007).
The observed antilipid peroxidation activity of ent-kaurene diterpenoid, melissoidesin (M) are in agreement with the ent-kaurene diterpenoids like leukamenin E, glaucocalyxin A, wangzaozi A, epinodisinol, epinodosin, rabdosin B, rabdosinate, lasiokaurin and oridonin isolated from Isodon racemosa and I. japonica var. galaucocalyx (Guoan Liu et al., 2006).

Fe$^{2+}$ and H$_2$O$_2$ are the most reactive species which damage almost every molecule in living cells. These reactive species have the capacity to join with the nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity (Moskovitz et al., 2002).

In the present study, prevention of deoxyribose degradation from reactive oxygen and hydroxyl radicals by melissoidesin (M) was observed significantly. The potent antioxidant activity of melissoidesin (M) may be attributed to the presence of electron donating groups. Similar activity was reported in Origanum vulgaris, Rosmarinus officinalis, Salvia officinalis and Thymus vulgaris (Dorman et al., 2003), Teucrium chamaedrys, T. montanum and T. polium (Tatjana Panovska et al., 2005) and Ocimum basilicum (Iris Hinneburg et al., 2006). Sideritis sipylea and Origanum sipyleum extracts prevent the deoxyribose degradation by scavenging the hydroxyl radicals (Nakiboglu et al., 2007).

In addition the observed activity of melissoidesin may be attributed to chelate the metal ions. The same condition was observed in the lyophilized aqueous extract of Ballota nigra showed ion binding capacity and can withdraw the iron ions and render them inactive (Vendula Vrchovska et al., 2007). The iron chelating activity correlated well with deoxyribose degradation inhibition was also reported in Ocimum basilicum (Iris Hinneburg et al., 2006). The same condition was observed in melissoidesin (M), the ent-kaurene showed both iron chelation and deoxyribose degradation activities in dose dependent manner. The result showed that there may be a correlation between iron chelation and deoxyribose degradation inhibition of melissoidesin (M).

In all antioxidant test systems studied the activity of melissoidesin (M) was lower than the positive control like BHT and BHA except DPPH and hydroxyl radical scavenging activities.
ANTIBACTERIAL ACTIVITY

The genus *Isodon* is a rich source of diterpenoids especially highly oxygenated *ent*-kaurenoids (Fujita and Node, 1984). Several diterpenes isolated from *Isodon* plants have shown antiviral (Olga Batista *et al.*, 1995), antitumor (Sun *et al.*, 2006) and antimicrobial (Turgut Kilic, 2006) activities. In the present study melissoidesin (M) an *ent*-kaurene diterpenoid isolated from acetone extract of the leaves of *I. wightii* was tested for antibacterial activity.

The result of antibacterial investigation on *I. wightii* was similar to that of *I. japonica* where 6, 7-seco-*ent*-kaurenoid, trichorabdial A and oridonin exhibited remarkable antibacterial activity against a gram negative bacterium *Helicobacter pylori* (Kadota *et al.*, 1997) and kaurene diterpenoids such as linearol, foliol, siderol, 7-epicandicandiol and epoxyisolinearol isolated from *Sideritis* species were reported to be antibacterial (Topcu *et al.*, 1999; 2001; 2002). 7α, 18-dihydroxykaur-16-ene isolated from *Sideritis athoa* showed remarkable antibacterial activity against *Enterococcus faecalis* and *Bacillus subtilis* (Fraga *et al.*, 2003) and rosthormins A-D isolated from *I. rosthornii* was found to showed potent antibacterial activity against *B. subtilis*, *Brevibacterium ammoniagenes*, *Streptococcus mutans* and *Staphylococcus aureus* (Kubo *et al.*, 2004). According to Kubo *et al.* (2004), α, β unsaturated ketone group in D-ring is a characteristic feature among many diterpenoids of *Isodon* species. The presence of this moiety is responsible for eliciting the biological activity such as antitumor and antibacterial activity. In the present study melissoidesin (M) an *ent*-kaurene diterpenoid showed antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* in the absence of α, β unsaturated ketone group in the D-ring. The observed antibacterial activity may be attributed to other reactive groups present in the structure.

Melissoidesin (M) isolated from *I. wightii* inhibited remarkably the growth of *S. aureus* (8.57mm) a gram positive bacterium followed by negative bacteria such as *P. aeruginosa* (7.25mm) and *E. coli* (6.75mm). Similar observations were made by Sun *et al.* (2006) where eriocalyxin B, an *ent*-kaurene diterpenoid from *I. eriocalyx* showed significant inhibitory activity against gram positive bacteria such as
Staphylococcus aureus, S. epidermidis and Streptococcus sanguis and gram negative bacteria such as Escherichia coli and Enterobacter gergoviae. Ent-kaurene diterpenoids like ent-1β-hydroxy-7α-acetyl-15β,16βepoxykaurene, sideroxol, 7-epicandicandiol, linearol, ent-7α,15β,18-trihydroxy-kaur-16-ene, ent-7α-acetyl,15,18-dihydroxy-kaur-16-ene, foliol, sideridiol isolated from the acetone extract of Sideritis stricta showed antibacterial activity against E. coli, S. aureus and Klebsiella pneumoniae (Turgut Kilic, 2006).

Other than ent-kaurenones several diterpenoids like norlindane such as multioorthoquinone isolated from Salvia multicaulis were reported to have strong activity against Enterococcus faecalis and P. aeruginosa and 2-demethyl multioorthoquinone was active against a gram negative bacteria E. coli (Ulubelen et al., 1997). Abietanes such as ferruginol, horminone, 7-acetyl horminone sugiol, 1-oxo-ferruginol isolated from Salvia blepharochlaena, S. viridis and S. candidissima showed inhibitory activity against S. aureus, S. epidermidis and B. subtilis (Ulubelen et al., 2000 and 2001).

**CYTOTOXICITY**

Several botanicals have been shown to exhibit antiproliferative and pro-apoptotic activity. There is extensive evidence that herbal mixture is strongly cytostatic and cytotoxic to a variety of tumor cell lines (Bigler et al., 2003). Phytochemical investigations for novel anticancer drugs from Lamiaceae have been long known with different types of diterpenoids and among all diterpenoids, ent-kaurene diterpenoids are promising candidate as an anticancer agent.

In the present study, ent-kaurene diterpenoid, melissoidesin (M) isolated from I. wightii under investigation showed cytotoxic activity against neuroblastoma (IMR-32) and lung cancer (A549) cell lines using MTT assay. Several ent-kaurene diterpenoids with potent cytotoxic activity was reported from group Isodon species.

Ent-kaurene diterpenoids, laxiflorins, maecrystal A and maecrystal P was isolated from the leaves of Leriocalyx var. laxiflora showed potent cytotoxic activity against K562, A549, and T24 (Human bladder carcinoma) cell lines. The observed cytotoxicity may be due to the presence of D ring. It has been suggested that there might
exist a relationship between the D-ring and cytotoxicity (Niu et al., 2002). The presence of D-ring in melissoidesin (M) may be responsible for observed cytotoxicity against neuroblastoma and lung cancer cell lines.

Melissoidesins (N-U) isolated from *I. melissoides* proved to be cytotoxic against human tumor cell line BGC-823 (Human stomach cancer) while melissoidesin (M) from the same plant was noncytotoxic (Zhao et al., 2004). In the present study melissoidesin (M) isolated from *I. wightii* showed cytotoxicity against neuroblastoma and lung cancer cell lines.

A group of ent-kaurene diterpenoids bisrubescensins A-C isolated from *I. rubescens* showed cytotoxicity against A549 cell with the IC$_{50}$ value of 0.54µM/ml (Huang et al., 2006). The activity of melissoidesin (M) was observed less than that of bisrubescensins against A549 and the IC$_{50}$ value were calculated as 6µM/ml. Meanwhile the cytotoxicity of melissoidesin (M) was more pronounced than that of alboatisins B isolated from *I. albopilosus* (Huang et al., 2007), the ent-kaurene showed the activity of 8.3µM/ml against A549.

The cytotoxic activity of melissoidesin (M) from *Isodon wightii* was similar to the results of Yu et al. (2007) where melissoidesin G isolated from *I. melissoides* was shown to inhibit human leukemia and acute myeloid leukemia cells via induction of apoptosis with the evidence of reactive oxygen production and nuclear fragmentation. and longikaurin B, and D, maeocrystal I, rosthorin A, rabdoterin E, ponidin, macrocalin B, xerophilusin A and N isolated from *Isodon xerophilus* showed potent cytotoxicity against K562, MKN45 (Adenocarcinoma) and HepG2 cell lines (Li et al., 2007).

**ANTICARCINOGENIC ACTIVITY**

Many plants growing in the wild or cultivated have been used as sources of different classes of useful chemicals, including natural antioxidants (Nakatani, 2002 and Madsen et al., 1996). Among the various medicinal herbs and plants, some species are endemic and important to that region since they may be used to produce raw materials as
well as finished pharmaceutical products containing antioxidant phytochemicals that provide significant health benefits.

Ent-kaurene diterpenoids represent a group of natural product in the genus *Isodon* have been reported to have various biological activities. In the present study the ability of melissoidsines (M) to protect cadmium chloride induced DNA damage in human peripheral blood lymphocytes was investigated using alkaline comet assay. The comet assay has been explored as a potential tool for detecting the antioxidant effect of food or nutrients (Noroozi *et al.*, 1998).

Cadmium is a common contaminant of hazardous waste sites and is released from sources such as fossil fuel combustion and municipal waste incineration and as a component of cigarette smoke (Maier *et al.*, 2000). Epidemiological studies identified lung, prostate and, to a lesser extent, kidney and stomach as primary targets for cadmium-induced tumorigenesis (Stohs and Bagchi, 1995). The formation of reactive oxygen species by cadmium suggests that DNA can also be taken into account as a potential target of this metal (Stohs *et al.*, 2000).

Cadmium disturbs the function of DNA repair enzymes produce DNA adducts by direct interaction with adenine or guanine and alter the expression levels of transcription factors implicated in apoptosis pathways form complexes with ascorbate and glutathione, inhibiting their scavenger capacity and therefore increase reactive oxygen species burden that could attack the DNA molecule (Asmuss *et al.*, 2000). The results obtained indicate that CdCl$_2$ can induce DNA damage in human lymphocytes. All the concentrations of CdCl$_2$ induced DNA damage in dose dependent manner. At 150µM/ml the tail movement was four times from the initial value as reported by Blasiak (2001). Hence in the present study 150µM/ml CdCl$_2$ was used to access the anticarcinogenic potential of melissoidsines (M). Melissoidsines (M) decreased extend of DNA damage evoked by CdCl$_2$. The DNA protecting ability of melissoidsines (M) in the present study was probably due to reactive oxygen species scavenging activity which may be associated with the presence of hydroxyl groups. The result are in agreement with the free radical scavenging efficiency of quercetin (Cotelle *et al.*, 1996) and free radical scavenging properties of phenolic diterpene, rosmarinic acid isolated from *Salvia officinalis* (Cristovao *et al.*, 2007).
According to Kapiszewska et al. (2005), DNA protecting activity of *Thymus piperella* and *Origanum heracleoticum* showed linear dependence between the DNA protection ability and the intercellular reactive oxygen species reduction. The same condition was observed in melissoidesin (M), the ent-kaurene showed DNA protecting activity with the reduction of reactive oxygen species produced by CdCl₂. The observed DNA protecting ability of melissoidesin (M) was also supported by Akhilesh Kumar and Sharmila Chattopadhyay (2007) in *Mentha spicata*. The methanol extract of *M. spicata* showed DNA protecting ability against H₂O₂ + UV-induced damage on pBS plasmid DNA.

The DNA protecting ability of melissoidesin (M) observed in the present study, i.e. significant reduction in the frequency of cells with DNA damage may be due to direct action of the melissoidesin (M) on CdCl₂ and prevent the formation of reactive oxygen species. This observation was concordance with the result of Siddique et al. (2008b) in antigenotoxic role of *Centella asiatica* extract against cyproterone acetate induced genotoxic damage in cultured human lymphocytes.

*Isodon wightii* containing ent-kaurene diterpenoid, melissoidesin (M) seemed to protect cells from DNA damage and reduce the side effects of CdCl₂. It thus proved possible to rank the potency of the phytochemical agents tested with high confidence.

**CONCLUSION**

The present study on *Isodon wightii* provides a reproducible, time saving and cost effective method for the mass multiplication. Phytochemical analysis and studies on the antioxidant, antibacterial, cytotoxicity and anticarcinogenic properties of the isolated compound melissoidesin (M) will provide an insight into the medicinal properties of *I. wightii*. 