In *cocculus hirsutus* 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 provide an important tool for mass multiplication, conservation and propagation of aromatic and medicinal plants (Khallafalla and Daffalla, 2008; Shahzad *et al*., 2011; Li *et al*., 2012).

The success of *in vitro* regeneration largely depends on a series of stages, each with a specific set of requirements. Some of these include physiological and developmental state of the explant, growth regulators, photoperiod and sequential subculture cycles (Fiore, *et al*., 2002; Yadav and Singh, 2011b). *In vitro* culture and in particular, nodal cultures have been successfully used to conserve medicinal plants (Beena *et al*., 2003). In menispermaceae family, nodal and leaf explants have been employed successfully for regeneration. *Cocculus hirsutus* twigs with three to four nodes were used as explants for regeneration (Meena *et al*., 2012). In the present study single node and leaf explants of *Cocculus hirsutus* responded favourably for regeneration.

Murashige and Skoog medium was found to be frequently used for *in vitro* culture in menispermaceae (Oselebe and Ene, 2007; Singh *et al*., 2009; Senarath, 2010; Kuo *et al*., 2011; Meena *et al*., 2012; Bhalerao *et al*., 2013). Woody plant medium was found to be used in *Tinospora cordifolia* (Raghu *et al*., 2006). In accordance with above observations, full strength MS medium showed maximum number of responding cultures from the node and leaf explants of menispermaceae plants. In correlation with the earlier reports, in the current study also MS medium was identified to be suitable for raising tissue cultures of *Cocculus hirsutus*. 
In *Cocculus hirsutus*, the nodal explants failed to respond morphogenetically to a hormone free MS medium. BAP at all concentration tested was more effective in shoot induction. Similar results were observed in early reports of *Cocculus hirsutus* (Meena et al., 2012). Shoot proliferation and multiplication are largely based on media formulations containing cytokinins as a major plant growth regulators (Mamidala and Nanna, 2009; Hoque, 2010). Some reports indicate the presence of cytokinin in the culture medium helped in the multiplication of shoots (Kumar et al., 2001). In the current study combination of BAP (0.5 mg/L) and TDZ (0.1 mg/L) induced maximum number of shoots (3.72) followed by combination of BAP and KIN (1.25) in MS medium. Similar result was reported by Kumari (2012) in *Tinospora cordifolia*. These results are in coherence with researches indicating the efficiency of cytokinins such as BAP, KIN and TDZ as useful agents in enhancing the auxiliary bud growth in medicinal plants (Borthakur and Singh, 2002). TDZ has been used successfully in *in vitro* culture to induce adventitious shoot formation and to promote axillary shoot proliferation in medicinal plants (Biswas et al., 2007). TDZ has been reported to enhance multiple shoot production from nodal explants of *Tinospora cordifolia* (Choudhary and Handique, 2013).

MS medium supplemented with combination of BAP and KIN along with additives like adenine sulphate and glutamine produced multiple shoots in *Cocculus hirsutus* (Meena et al., 2012). In the present study two to three shoots are produced in combination of BAP and KIN without additives.

Enhancement of shoot elongation was achieved by subculturing the shoots in full strength MS medium supplement with cytokinin (BAP, TDZ) in combination with GA$_3$ (0.2 mg/L). Among the cytokinins and GA$_3$ combination tested, maximum shoot length (3.92 cm) were obtained on MS medium supplemented with BAP (0.3 mg/L), TDZ (0.1 mg/L) and GA$_3$ (0.2 mg/L) followed by BAP (0.3 mg/L and GA$_3$ (0.2 mg/L) which produced 3.26 cm length.
shoots. In previous report, lower level of BAP (0.25 mg/L) favored the elongation of shoots in *Cocculus hirsutus* (Meena *et al*., 2012). Similar effects of lower concentrations of cytokinin on shoot elongation have been reported also by Gayathri *et al*. (2009) and Uranbey *et al*. (2010).

Shoot organogenesis from callus cultures can be used as an effective method for multiplication of medicinal plants (Reddy *et al*., 2001). In the present study callus mediated shoot organogenesis was chosen as an alternative method for shoot multiplication.

The influence of growth regulator on callus development from leaf explants was investigated using various levels of 2, 4-D, BAP and NAA. Among the auxin tested, 2, 4-D with L-Glutamine induced white callus from the leaf explant and combination of BAP and NAA initiated callusing from the explants but could not sustain its further growth. Addition of activated charcoal to the BAP and NAA supplemented medium proved good for callus growth. Khalilsaraie *et al*. (2011) reported MS medium supplemented with 2, 4-D (1mg/L) produced Whitish friable callus. These results are in agreement with Bhalerao *et al*. (2013) and Singh *et al*. (2009) on *Tinospora cordifolia*, where callus induction was obtained when 2, 4-D alone and combination of BAP and NAA were added to the cultured media. In contrast, the MS medium supplemented with combination of 1 mg/L of BAP and 0.5 mg/L TDZ induced callus from leaf explant of *Stephania tetrandra* (Kuo *et al*., 2011).

The shoot regeneration from the callus in plants belonging to menispermaceae family indicates the important role of BAP in shoot initiation, either individually or in combination with other growth regulators (Gururaj *et al*., 2007; Sing *et al*., 2009 and Kuo *et al*., 2011). In the present study, leaf derived callus of *Cocculus hirsutus* was subcultured in full strength MS medium with combination of BAP and GA₃, BAP at 0.7 mg/L and GA₃ at 0.5 mg/L developed shoots.
Induction of roots at the base of *in vitro* grown shoots is essential and indispensable step to establish tissue culture derived plantlets to the soil. Rooting of *in vitro* produced shoots was best on half-strength MS medium supplemented with IBA, which has also been reported for some other medicinal plants (Senarath *et al*., 2007). It is evident that auxins are essential for root induction and in the present study two auxins IBA and IAA was tested and IBA was found to be effective. The maximum frequency of root formation and the number of roots formed was significant in half-strength MS medium supplemented with IBA (0.5mg/L). Similar result was reported in *Coscinium fenestratum* (Senarath, 2010).

Hardening is crucial step prior to transplantation of plantlets from *in vitro* to *in vivo* phase. Generally, higher sophistication (controlled humidity, temperature and accurate potting mixture) is used for higher plantlet survival. In the present study acclimatization of *Cocculus hirsutus* has been achieved easily with 70% survival by way of simply maintaining the plantlets at 25-28 °C with 70-80% relative humidity in the potting media containing garden soil, farmyard and sand in the ratio of 2:1:1. Similar results have also been obtained in *Tinospora cordifolia* and *Cocculus hirsutus* (Raghu *et al*., 2006; Meena *et al*., 2012).

**Phytochemical analysis of *in vivo* and *in vitro* plantlets**

*Cocculus hirsutus* belonging to the menispermaceae family has attracted considerable attention because of its various biological activities including antimicrobial, antifungal and anti-inflammatory effects as well as its clinical use in the treatment of various diseases. Alkaloids are the major chemical constituents of *Cocculus* species (Bhakuni *et al*., 1970; Tahir *et al*., 1991; Ahmad and Iqbal, 1992).

Kalirajan *et al*. (2012) reported the methanol and water extracts of *cocculus hirsutus* possessed alkaloids, steroids, saponins and tannins. In the present investigation also the
methanol and water extracts of in vivo and in vitro leaves of Cocculus hirsutus expressed positive results for various phytochemical.

Madhavan et al. (2010) revealed the presence of alkaloids, carbohydrates, glycosides and saponins in alcohol (ethanol) and aqueous extracts. The benzene extract revealed the presence of fixed oil and fats in Cocculus hirsutus. The present study results were directly coincided with the above results.

Chloroform extract have not show any secondary metabolite in Cocculus hirsutus (Madhavan et al., 2010). In the present study the in vivo and in vitro leaves revealed the presence of alkaloids in chloroform extract. The variation in the phytoprofile may be due to the locality/ habitat difference. In comparison to the previous work, methanol and water extracts of in vivo and in vitro leaves showed the presence of maximum number of secondary metabolites. Therefore results indicate that the extracts of Cocculus hirsutus might have the possibility of containing components of biological activity.

ANTIOXIDANT STUDIES

Natural products research is an important aspect of the drug discovery process, and a modular approach towards screening with sophisticated methodologies to evaluate the extracts, fractions and pure compounds for biological activity has enabled identification of biologically active compounds. Several herbs, which are used in our daily diet, are very rich source of antioxidants. In take of natural antioxidant has been associated with reduced risk of cancer; cardiovascular diseases, diabetes and other diseases associated ageing (Halliwell, 1994).

All aerobic organisms have evolved with an antioxidant defence system to counteract oxidative stress from ROS (Zou et al., 2008). The antioxidant defense can be obtained through the food and endogenous antioxidants. Endogenous antioxidants constitute the
enzymes catalase, peroxidise, etc. the molecules that are capable of scavenging the ROS and RNS are definite to possess an inherent biological effect like anticancer, antimicrobial, anti-inflammatory, antidiabetic and soon. Therefore identifying a compound or extract to contain antioxidant potential ensures the presence of some biological activity. The current study has been done with this view and antioxidant potential of *in vitro* and *in vivo* leaves of *Cocculus hirsutus* and endogenous antioxidant content of the leaves were analysed.

Catalase is present in the peroxisome of aerobic cells and is very efficient in promoting the conversion of hydrogen peroxide to water and molecular oxygen (Rahman, 2007). Catalase enzyme converts hydrogen peroxide to non radical form and function as natural antioxidant. Catalase is primary hydrogen peroxide scavenger in peroxisomes and the mitochondria (Zolfaghari et al., 2010). *Terminalia bellirica*, an important medicinal plant was investigated for the presence of the antioxidant enzyme and was reported with a catalase activity of 501.8 ±42.079 units/gm of tissue (Mary et al., 2013). There are reports on the presence of catalase in the tuber, young leaf and mature leaf of *Amorphophallus commutatus* (Kavithakrishna et al., 2012). The current study reports a catalase content that is almost 40 percent lesser than Mary et al. (2013) and 30 percent higher that Kavithakrishna et al. (2012). JayaChitra and Padma (2012) reported that the white and blue flowered leaves of *Clitoria ternatea* showed the catalase activity of 133.63 ± 0.961 U/gm of tissue and 110.39 ± 1.969 U/gm of tissue respectively, which was comparatively lesser than the current report.

The catalase activity in the present investigation is in accordance with the activity produced by grapes (193.59 U/gm of tissue) for *in vitro* leaves and with oranges (106.54 U/gm of tissue) for *in vivo* leaves (Rani et al., 2004). The level of the active antioxidant enzymes of the leaf samples of the plant *C. zedoaria* (Christm.) and was found to be
58.4± 1.5 U/mg catalase. The present study indicates that Cocculus hirsutus has considerable catalase activity in the in vivo and in vitro leaves.

Polyphenol oxidase (PPO) is a stress marker enzyme, produced as protective measure against diseases. The antioxidant activity of polyphenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers. Antioxidants inhibiting the oxidation of organic molecules are very important, not only for food preservation, but also for the defence of living systems against oxidative stress (Raghuveer et al., 2011). The activity of PPO in the present study shows 0.207±0.002 U/gm of tissue for in vivo leaf and 0.214 ± 0.001 U/gm of tissue for in vitro leaf. The activity of PPO in the present study is higher than Terminalia bellirica leaves and fruits (Mary et al., 2013) and lower than Amorphophallus commutatus tuber, young leaf and mature leaf (Kavitha Krishna et al., 2012).

Jayachitra and Padma (2012) reported that the white and blue flowered leaves of Clitoria ternatea showed the polyphenol oxidase activity of 0.322 ± 0.011 U/gm of tissue and 0.260 ± 0.010 U/gm of tissue respectively. So in this study, the in vivo and in vitro leaves of Cocculus hirsutus showed lower polyphenol oxidase activity compared to white and blue flowered leaves of Clitoria ternatea.

Rani et al. (2004) reported the ascorbic acid oxidase activity of goose berry, grapes, orange and tomato with 0.018, 0.005, 0.046 and 0.007 U/gm of tissue respectively. The present study indicates that Cocculus hirsutus has considerable ascorbic acid oxidase activity in the in vitro leaves.

Tingentsai and Taatsai leaves (leafy vegetables) showed the ascorbic acid oxidase activity of 0.008 and 0.049 U/gm of tissue respectively (Shimada and and Sanae, 2008). In the current report the ascorbic acid oxidase activity is lower than both the leafy vegetables. In
comparison, the concentration of ascorbic acid oxidase enzyme was low in the *in vivo* and *in vitro* leaves of *Cocculus hirsutus* than catalase and polyphenol oxidase.

The stable radical DPPH has been used widely for the determination of primary anti-oxidant activity (Katalinic *et al.*, 2004). The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of anti-oxidants (Ara and Nur, 2009). The present study indicates that the IC$_{50}$ value of methanol extract of *in vivo* and *in vitro* leaves are quite significant when compared to the standard which may be attributed to its poor proton donating ability (Voravuthikunchai *et al.*, 2009).

The radical scavenging activity of *in vitro* and *in vivo* leaf extracts of *Cocculus hirsutus* against the oxidant DPPH was comparatively higher in *in vivo* leaf extract. The report from the previous researchers on radical scavenging activity and comparing different species from the same family demonstrated the presence of this property.

Ethyl acetate, petroleum ether and methanol extracts of *Cocculus hirsutus* leaf exhibited IC$_{50}$ values of 287.960 μg/ml, 1701.19 μg/ml and 257.419 μg/ml respectively (Nizam *et al.*, 2013). In the present study the methanol extracts of *in vivo* and *in vitro* leaves showed lower IC$_{50}$ value, which indicate higher antioxidant activity. Similarly benzene and chloroform extracts of *in vivo* and *in vitro* leaves of *Cocculus hirsutus* extract revealed lower IC$_{50}$ value compare to petroleum ether and ethyl acetate extracts.

The whole plant of *Stephania japonica* showed the IC$_{50}$ value of 422.321 μg/ml in ethanol extract. The methanol extracts of *Tinospora crispa* aerial parts showed the IC$_{50}$ value of 90.074 μg/ml (Nizam *et al.*, 2013). In the present study, the *in vivo* and *in vitro* leaves of *Cocculus hirutus* extract showed lower IC$_{50}$ value than above extracts.

The study carried out by Ramya and Lakshmidevi (2010) has shown the DPPH radical scavenging activity of *T. cordifolia* leaf extract (Methanol) with an EC$_{50}$ value of 0.9
mg/ml. Mon et al. (2011) reported that the free radical scavenging activity (DPPH) of ethanol extract of C. hirsutus leaf to be significant among the three tested plants. The EC$_{50}$ value of C. hirsutus leaf extract was 10.68 μg/ml. The difference in the EC$_{50}$ value can be attributed to the distribution of secondary metabolites that may fluctuate between different plant organs (Lissiewska et al., 2006).

The methanolic leaf extract of Coscinium fenestratum showed maximum DPPH scavenging activity at a concentration of 512μg/ml and minimum was at 2μg/ml (Goveas et al., 2013). The ethanol extract of Cocculus hirsutus aerial parts scavenged 94.45% DPPH free radical at 100 μg/ml concentration (Panda et al., 2011). In this study methanol fraction of in vitro and in vivo leaves exhibited significant DPPH radical scavenging activity.

The methanolic stem extracts of Arcangelisia flava, Coscinium blumeanum and Fibraurea tinctoria showed the DPPH radical scavenging activity with an EC$_{50}$ value of 25.7 ± 1.7μg/ml, 50.1 ± 0.6 μg/ml and 83.6 ± 1.8 μg/ml respectively (Keawpradub et al., 2005). When compared the methanol extract of in vitro and in vivo leaves of C. hirsutus possessed significant antioxidant activity than Coscinyium blumeanum and Fibraurea tinctoria stem extracts and less antioxidant activity than Arcangelisia flava stem extract.

The chloroform stem extracts of Arcangelisia flava, Coscinium blumeanum and Fibraurea tinctoria showed the DPPH radical scavenging activity with an EC$_{50}$ >100 μg/ml, 55.4 ± 0.3 μg/ml and 78.8 ± 1.6 μg/ml respectively (Keawpradub et al., 2005). The chloroform leaf extract of in vitro and in vivo leaves of C. hirsutus showed less antioxidant activity than Coscinium blumeanum and Fibraurea tinctoria stem extracts and significant antioxidant activity resulted in Arcangelisia flava stem extract.

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as highly damaging species in free radical pathology,
capable of damaging almost every molecule found in living cells (Hochestein and Attalah, 1988).

The ethanol extract of *C. hirsutus* aerial parts in different concentration produced a dose dependent scavenging of OH⁻ radical (Panda *et al.*, 2011). In the current study benzene, chloroform and methanol extracts of *in vivo* and *in vitro* leaves showed significant scavenging of OH⁻ radical followed by methanol > benzene > chloroform in descending order. This effect may be due to the presence of phytoconstituents on these extracts.

The 70% methanol extract of *T. cordifolia* was found efficient in scavenging of Hydroxyl radical. The IC₅₀ value for the extract was found to be 128.86 ± 4.07μg /ml (Nikhil *et al.*, 2012). In the present study benzene, chloroform and methanol extracts of *in vivo* and *in vitro* leaves showed significant IC₅₀ values than the above mentioned report.

Nitric oxide plays a vital role in various inflammatory processes. Higher levels of these radical are toxic to tissue and contribute to the vascular collapse. Hyper level expression of nitric oxide radical is associated with various carcinoma and ulcerative colitis. The toxicity of nitric oxide increases when it reacts with superoxide radical forming highly reactive peroxy nitrate anion (ONOO⁻). Reactive oxygen species, nitric oxide is implicated in inflammation, cancer and other pathological condition (Moncada *et al.*, 1991).

The ethanol extracts of *C. hirsutus* aerial parts in different concentration scavenged nitric oxide radicals with the percentage of scavenging ranging between 64 and 92 (Panda *et al.*, 2011). In coherence with the above report the methanol extract of *in vitro* leaves of *cocculus hirsutus* posses significant nitric oxide scavenging activity.

The 70% methanol extract of *T. cordifolia* was found efficient in scavenging nitric oxide radical. The IC₅₀ values for extract was 51.98 ± 4.80 μg /ml (Nikhil *et al.*, 2012). Aqueous and ethanolic extract of *Tinospora cordifolia* showed that IC₅₀ values 239.00 μg/ml.
and 254.06 μg/ml respectively (Devprakash et al., 2012). Our result showed that the methanol extract of \textit{in vivo} and \textit{in vitro} leaves had significant scavenging of nitric oxide radicals and exhibited an IC$_{50}$ value similar to 70% methanol extract of \textit{T. cordifolia}. Benzene, chloroform and methanol extracts of \textit{in vivo} and \textit{in vitro} leaves of \textit{Chirsutus} exhibited IC$_{50}$ values lower than aqueous and ethanolic extract of \textit{Tinospora cordifolia}.

Reducing power is associated with its anti-oxidant activity and may serve as a significant reflection of the anti-oxidant activity (Oktay \textit{et al.}, 2003). In the present study, the results indicate that the reducing ability of the extracts increased with the concentration. Among all the extracts tested for their reducing abilities methanol extract of \textit{in vitro} and \textit{in vivo} leaves of \textit{Cocculus hirsutus} was significant. The reducing power of \textit{in vivo} and \textit{in vitro} extracts in the descending order was methanol> benzene> chloroform. The reducing capacity of ascorbic acid was found to be higher than the extracts at each concentration points. There exists a direct correlation between antioxidant activities and reducing capacity of the plant extracts (Gao \textit{et al.}, 2000). A study carried out by Ramya and Lakshmidevi (2010) and Praveen \textit{et al.} (2012) has shown the reducing power activity of methanolic leaf extracts of \textit{T. cordifolia} increased with the absorbance.

In the current study, Total antioxidant activity of \textit{in vivo} and \textit{in vitro} extracts was as mentioned in descending order methanol> benzene> chloroform. The results indicate that the activity of the extracts increased with the concentration. Smilarely, Nunes \textit{et al.} (2013) reported, total antioxidant activity of ethanol extracts of red propolis increased with the absorbance.

Analysis of the \textit{invitro} radical scavenging capacity of various free radicals and endogenous antioxidant content of \textit{in vitro} and \textit{in vivo} leaves of \textit{Cocculus hirsutus} reveal that the \textit{in vitro} leaves out performed \textit{in vivo} leaves by possessing significant activity. This could
be because the *in vitro* leaves arise in a controlled environment. This results emphasis on the importance of tissue culture in producing pharmaceuticals through suspension culture, thereby overcoming the problem of exploitation of any plant species.

**ANTIBACTERIAL ACTIVITY**

The emergence of antibacterial resistance among bacteria has induced a compulsory need for search and identification of newer, novel and safer antibacterial agents. Over the last forty years intensive efforts have been made to discover clinically useful antibacterial drugs (Sashikumar *et al.*, 2003). Over 50% of all modern clinical drugs are of natural product orgin (Ates, 2003). Recently, a number of plants have been reported for antibacterial properties across the world (Koshi Philip *et al.*, 2009).

Benzene, chloroform, methanol and water extract of *in vivo* and *in vitro* leaves of *Cocculus hirsutus* were analysed for antibacterial activity against *Pseudomonas aeruginosa, Escherichia coli, Klebsilla pneumonia, Staphylococcus aureus and Bacillus sp*. In *in vitro* leaf extracts maximum zone of inhibition was observed in water extract against *E.coli* followed by methanol extract against *S.aures* and lower zone of inhibition was identified in benzene extract against *E.coli* followed by chloroform extract against *K.pneumoniae*. Among *in vivo* leaf extracts maximum zone of inhibition was observed in water extract against *E.coli* followed by methanol extract against *S.aures* and lower zone of inhibition was obtained in benzene extract against *E.coli* and *P.aeruginosa* followed by chloroform extract against *K.pneumoniae*.

The zone of inhibition exhibited by *in vitro* and *in vivo* leaves were almost similar except the two organisms *Pseudomonas aeruginosa* and *Bacillus sp*. Both organisms were inhibited significantly by *in vitro* extracts. This again provides us with evidence, that *in vitro*
grown leaves under controlled condition hold better phytochemical content. This emphasis the importance of tissue culture in obtaining secondary metabolites for medical applications.

The methanol and aqueous leaf extract of *Cocculus hirsutus* was identified to be effective against *E.coli*, *vibrio cholera*, *Staphylococcus aureus* (Kalirajan *et al.*, 2012). The current study has exhibited similar results exhibiting antibacterial activity against *E.coli* and *Staphylococcus aureus* but in the current study *in vitro* leaf samples were also analysed which exhibited better activity.

Abiramasundari *et al.* (2011) studied the antibacterial activity of different solvent extracts of *cocculus hirsutus* leaves (*in vivo*) against five human pathogens Viz., *B. subtilis*, *E.coli*, *S.aureus*, *S. dysentriae* and *K.pneumoniae* and reported various degree of zone of inhibition. Similar to this observation, in the present study, the benzene, chloroform, methanol and water extracts of *in vivo* and *in vitro* leaves of possessed antibacterial activity against other tested organisms except *K.pneumoniae*.

The chloroform root extracts of *Cocculus hirsutus* inhibited the pathogenic bacteria *Pseudomonas aeruginosa* and *Bacillus cereus*. The inhibition against *Staphylococcus aureus* was moderate and the remaining stains *E.coli* and *K.pneumoniae* had no activity and methanol and water extracts also had no activity against the above mentioned stains (Jeyachandran *et al.*, 2008). In the current study chloroform, methanol and water extracts of *in vivo* and *in vitro* leaves significantly inhibited all the tested organisms.

Water and methnolic leaf extracts of *C.fenestratum* were studied for antibacterial activity against four bacterial stains, namely *S.aureus*, *E.coli*, *B. subtilis* and *P.aeruginosa*. The methanol and aqueous extracts of *C.fenestratum* leaves showed maximum zone of inhibition against *S.aureus* and *B. subtilis* respectively (Goveas *et al.*, 2013). Similar results
were obtained in *Cocculus hirsutus* leaf with methanol extract and contradictory results were obtained with aqueous extract in the current study.

Methanolic extract of *in vitro* grown plants and callus of *Tinospora cordifolia* showed a broad spectrum of activity against all tested stains at 10-20 µg/disc. Highest zone of inhibition was observed in *Staphylococcus aureus* and least activity was observed in *S. typi* (Kumari *et al.*, 2012). In the present study *in vitro* leaves showed significant zone of inhibition against *Staphylococcus aureus* and comparatively less significant activity was observed against *P. aeruginosa*.

**QUALITATIVE AND QUANTITATIVE PHYTOCHEMICAL ANALYSIS OF *COCCLUS HIRSUTUS* FRUIT**

The fruit of *Cocculus hirsutus* are small, dark purple pigmented. The review of literature indicated the absence of phytochemical and pharmacological reports on this fruit. But the Jhabua, Khargone and Dhar tribes has been using this fruit for various ailments and especially to treat jaundice (Samvatsar and Diwanji, 2000). The local practitioners of medicine were found to use this fruit in various preparations. Hence inorder to understand the phytochemical possession and biological activity the fruits were analysed.

In the presence of ferric chloride and aluminium chloride, the fruit extract showed brown colour and dark blue colour respectively, which confirmed the presence of flavonoids. *Cocculus hirsutus* fruit extract was stable after adding 2M Hcl and the colour changed to green after adding 2M NaOH, which confirmed the presence of anthocyanin. However, the colour change principally depends on pH, nature of the environment in which the compound occurs, as well as their substitution pattern (Finar, 2008). Similar results were observed in the fruits of *solanum nigrum*, *Kirganelia reticulata*, *Syzigium cumini* and *Opuntia dilleni* (Sivasankar *et al.*, 2011) and the fruit skin of *Pyrus communis* (Huang *et al.*, 2012).
In vitro Propagation, phytochemical screening and biological activity of Cocculus hirsutus (L.) Diels.

In the current study the maximum absorption of the compound 1 and compound 2 in visible region were found at 527 nm and 530 nm respectively. Our results were accordance with the reports of Siegelman and Hendricks, 1958. So these compounds may be a Cyanidin compounds. Anthocyanins are characterized by two absorptions: Band I, 475-560 nm (visible region) and band II, 275-280 nm (UV region). The wavelength range, 505-535 nm is one at which the conjugated bond in anthocyanins structures absorbs light, which are the basis for its bright red, blue and purple colours of fruits and vegetables, (Ganiyat et al., 2011).

**Total Phenol**

Phenols consist of flavonoids, phenolic acids, and other non-flavonoid phenols, which exhibit a number of chemical and biological activities. Total poly phenol content in Cocculus hirsutus was 326.66 ± 3.05 mg GAE/g. the fruit of T. cordifolia was found to have phenolic contents of 2.4, and 3.2 mg gallic acid equivalent (GAE)/g fresh weight in methanol and 80% ethanol fractions, respectively (Khan et al., 2011). Total polyphenol contents in T. cordifolia fruit extracts were lower than Cocculus hirsutus fruit extract. Water and ethanol extract of Morus nigra fruit possessed phenolic contents of 118.84±0.93 mg GAE/100g and 90.26±0.92 mg GAE/100g respectively (Kostic et al., 2013). Organic and Conventionally cultivated blueberry extracts had a phenolic content of 319.3 mg GAE/100g and 190.4 mg GAE/100g respectively. The total phenolic contents of Rubus anatolicus, R. caesius and R. raddenus fruit extracts were 688 ± 5.59, 474 ± 3.77 and 584 ± 4.42 mg GAE/100g FW respectively (Aezam and Nasrin, 2013). The results of the current report indicate the presence of significant quantity of phenol in Cocculus hirsutus fruit. The phenolic compounds play a vital role in plant defense mechanisms, to counteract reactive oxygen species, in order to survive and prevent molecular damage, and damaging by microorganisms, insects and herbivores (Kadir et al. 2009).
Total flavonoids

The *C. hirsutus* L. fruit extract showed significant total flavonoid content of 260 ± 20 mg/g of fresh weight. The flavonoid content in sweet cherries of different genotype cultivars samples ranged between 0.42 ± 0.13 and 5.63 ± 0.05 mg/g (Prvulović et al., 2011). Devi et al (2011) reported that the flavonoid content of acidified methanol extract of *Pithecellobium dulce* fruit pericarp was 6.2 mg/g. Similar to the phenol content the flavonoid content of *Cocculus hirsutus* fruit was significant in the current report.

Total anthocyanin

Total anthocyanin content in (different cultivars like Lambert, Bing, Stella, Compact, Napoleon and Petrovka) sweet cherries varied from 29 to 62 mg/100 g of fresh weight. The exception was Petrovka variety, with twice the amount of total anthocyanins, 62 mg/100 g of fresh weight (as cyanidin-3-glucoside), when compared to other cultivars (Mozetic et al., 2002). The 19 strawberry genotypes tested for anthocyanin contents by the pH differential method. BC92-20-85 fruits had the highest total anthocyanin content of 43.7 ± 5.3 mg/100g and unreleased advanced line SJO001-99 had the lowest anthocyanin content 6.3 ± 1.2 mg/100g (Samir and Elodie, 2009). Total anthocyanin content of *Cocculus hirsutus* in the present investigation is significant.

The phenol, flavonoid and total anthocyanin contents of *Cocculus hirsutus* were found to be significant. These phytochemicals are underlying principle in various biological activities the plant possesses. Hence, further studies to understand the bioactivity has been carried out.

ANTIOXIDANT STUDIES OF FRUIT EXTRACTS

DPPH is a stable free radical, the antioxidant activity of plant extract was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH (Koleva et al., 2002). The absorbance decreases as a result of a colour change from purple to
yellow due to the power of hydrogen donating ability (Blois, 1958). The IC\textsubscript{50} value of DPPH radical scavenging of the red purple coloured fruits of *Chrysophyllum cainito, Gaultheria shallon, Malpighia glabra, Myrciaria cauliflora* and *Sambucus caerulea* fruits were $7.9 \pm 0.3 \ \mu g/ml, 5.9 \pm 0.3 \mu g/ml, 13.9 \pm 1.3 \mu g/ml, 6.2 \pm 0.7 \mu g/ml and 16.9 \pm 0.6 \mu g/ml$ respectively. These fruits were confirmed for the presence of anthocyanin (Einbond *et al.*, 2004). *Syzygium cumini* fruit skin contains anthocyanin and the IC\textsubscript{50} values of DPPH scavenging in the water extract was $168 \mu g/ml$ (Banerjee *et al.*, 2005). The *C.hirsutus* fruit extract contain significant DPPH scavenging activity when compared to *Syzygium cumini* fruit skin extract but *Chrysophyllum cainito, Gaultheria shallon, Malpighia glabra, Myrciaria cauliflora* and *Sambucus caerulea*, possessed significant DPPH radical scavenging activity when compared to *C.hirsutus* fruit.

ABTS also forms a relatively stable free radical, which decolorizes in its non-radical form (Shirwaikar *et al.*, 2006). ABTS$^{o+}$, a nitrogen centered cation radical generated by oxidation of ABTS in the presence of potassium per sulphate prior to reaction with putative antioxidants (MacDonald-Wicks *et al.*, 2006). The IC\textsubscript{50} value of ABTS scavenging of *C.hirsutus* extract was not significant when compared to black raspberry ethanol extract and ethanol extract of anthocyanin from calyx fruit of roselle (Jeong *et al.*, 2010; Yang *et al.*, 2012). The presence of significant ABTS radical scavenging potential in the fruits of two jabuticaba varites (Paulista and Sabara) are reported (Lima *et al.*, 2011).

The available nitric oxide radical is linked with various carcinomas and inflammatory conditions. The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract directly competes with oxygen to react with nitric oxide and thereby inhibits nitrite formation. Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Jorgensen *et al.*, 1999). The nitric oxide scavenging activity of
flavonoids and phenolic compounds are identified by Jagethia et al. (2004). The scavenging of nitric oxide by anthocyanin extract of berries increased in a dose-dependent manner (Nikhah et al., 2009). In the present study nitric oxide scavenging effect of C. hirsutus fruit extract was significant. Pergola et al., (2006) reported nitric oxide biosynthesis inhibition by blackberries anthocyanin extract. Their studies reported that a part of anti inflammatory activity of blackberry extract was related to nitric oxide production inhibition by cyaniding-3-glucoside that it is major anthocyanin in blackberry extract.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Blazovics et al., 2003). Reducing power of anthocyanin extract from Morus alba fruit showed the absorbance of 0.005 to 0.025 at 110-341 µg/ml concentration. The reducing power of red sorghum bran anthocyanin was 0.984 at 100 µg/ml concentration (Suganyadevi et al., 2012). Kutlu et al. (2011) reported that the acidified methanol extract of Morus nigra’s reducing power increased with increase in concentration of extract. In the present study, the data showed that reducing power of the extract increased with increased concentration of anthocyanin extracts. So C. hirsutus fruit anthocyanin extract has more significant antioxidant activity than Morus alba and Morus nigra extracts and less antioxidant activity than red sorghum bran anthocyanin.

The reducing power of berries anthocyanin extract was potent and the power of the extract increased with quantity of sample of for the measurements of the reductive ability, the transformation of Fe³⁺ to Fe²⁺ was investigated in berries anthocyanin extract (Oyaizu, 1986). Baea and Suh (2007) studied antioxidant activities of five different mulberry cultivars in Korea, and has reported that high value of reducing power was an indication that some compounds in mulberry extract could be an electron donors which could react with free
radicals and convert it in to more stable products thereby terminating free radical chain reactions.

A modified thiobarbituric 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., 2002). The presence of Cocculus hirsutus fruit extract 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 percentage of inhibition of linoleic acid peroxidation for different fractions of Dendrobium sonia anthocyanin was between 37 to 54 %. It was found that crude extracts and water layer contained comparable values of lipid peroxide inhibition activity (Shafazila and Lee, 2011). In contrast, Cocculus hirsutus fruit anthocyanin extract possessed comparatively less lipid peroxide inhibition activity.

The anthocyanin extracts of Syzygium cumini fruit skin from 7 days to 6 months after drying were tested and the IC$_{50}$ values for the inhibition of lipid peroxidation were 222 μg/ml in on 7 days after drying and 268 μg/ml in fruit skin at 6 month after drying (Banerjee et al., 2005). These values indicate that Cocculus hirsutus possessed significant lipid peroxidation in fruit extract.

Chelating agents may inhibit radical generations by stabilizing transition metals, consequently reducing radical damage. Some phenolic compounds exhibit antioxidant activity through the chelation of metal ions (Zhao et al., 2008). The sorghum anthocyanins
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exhibited the highest activity of 85.2% at 100 μg/ml whereas 48.3% inhibition was noted at 1 μg/ml, respectively. The acidified methanol extract of the sorghum anthocyanins had the highest chelating capacity than BHT and ascorbic acid (Suganyadevi et al., 2012). Kutlu et al. (2011) reported the acidified methanol extract of Morus nigra’s fruit anthocyanin showed metal chelating activity of 85% at 100 μl (10 gm/100 ml). In this study cocculus hirsutus fruit extract showed chelating capacity of 15.07±1.08 % at 100μg/ml concentration. However, the chelating capacity was found to be less when compared to the standard EDTA. Anthocyanins are reported to be good chelators for metal ions, which are well known catalyzers for free radical forming reactions (Dangles et al., 2000).

The spectrophotometric measurement of Total antioxidant capacity (TAC) is based on the reduction of Mo (VI) to Mo (V) by antioxidant compound and the formation of green phosphate / Mo (v) complex at acidic pH (Prieto et al., 1999). Increase in absorbance indicates increase in total antioxidant capacity. Crude ethanol extracts of wampee fruit peel showed a good total antioxidant activity, which was concentration-dependent (Nagendra Prasad et al., 2009). The cocculus hirsutus fruit extract exhibited significant activity and its absorbance increased with increase in concentration. The fruit of the two jabuticaba varieties (Paulista and sabara) presented high antioxidant activities of 113.53 ± 18.8 mg/g, 127.56 ±3.23 mg/g respectively. These values were lower than standard rutin (290.89 ± 15.7 gm/g) (Lima et al., 2011).

Hydroxyl radicals generated by the Fenton reaction are known to cause oxidatively induced breaks in DNA strands to yield fragmented forms. The anthocyanins showed significant reduction in the formation of nicked DNA and increased native form of DNA. Quercetin effectively protected DNA strand scission from tertiary-butyl hydroperoxide (Sestili et al., 1998). In biological systems metal binding can occur on DNA leading to
partial site-specificity hydroxyl radical formation. Anthocyanins are potential protecting agents against the lethal effects of oxidative stress and offer protection of DNA by chelating redox-active transition metal ions. Mas et al., (2000) suggested that anthocyanins have the ability to stabilize DNA triple-helical complex. Kumar et al. (2007) reported that oxidative modification of DNA has been suggested to contribute to aging and various diseases including cancer and chronic inflammation. Our results are similar with Red Sorghum (Sorghum bicolor) bran on the DNA damage protecting activity of anthocyanins (Suganyadevi et al., 2012).

ANTIBACTERIAL ACTIVITY

Acidified methanol extract of Cocculus hirsutus fruit was analysed for antibacterial activity against Pseudomonas aeruginosa, Escherichia coli, Klebsilla pneumonia, Staphylococcus aureus and Bacillus sp. In the fruit extract, maximum zone of inhibition was observed in Bacillus sp followed by E.coli. > S. aureus >P. aeruginosa > K. Pneumonia and lower zone of inhibition was identified in K. Pneumonia followed by the order of Bacillus sp. < E.coli < S. aureus < P. aeruginosa.

Liepina et al. (2013) studied the antibacterial activity of aqueous and ethanolic fruit extracts of Black chokeberry and Rowanberry against four pathogens namely Bacillus cereus, Staphylococcus aureus, Eschericha coli and Pseudomonas aeruginosa. These extracts inhibited all the tested stains except E.coli and better results was obtained in ethanol extract. In both the fruit extracts (ethanol), the highest zone of inhibition was obtained in Bacillus cereus and least activity was observed in S.aureus. In the current study acidified methanolic fruit extract significantly inhibited all the tested organisms and the maximum and minimum zone of inhibition was similar to the above report.
Methanol, ethyl acetate and dichloromethane extracts of *Syzygium cumini* fruit peel was investigated against fourteen microorganisms. Methanol extracts showed maximum antimicrobial activity potency against all the test microorganisms (Priya *et al.*, 2013). In the present study acidified methanolic fruit extract significantly inhibited all the tested organisms.

Burdulis *et al.* (2009) reported that the Bilberry and Blue berry extracts showed strong zone of inhibition against Gram-negative bacteria of *Escherichia coli* and *Pseudomonas aeruginosa* and Gram-positive bacteria of *Bacillus* *sp* and *S.aureus*. Similarly, in the current study fruit extract inhibited both the Gram-negative and Gram-positive strains.

**CYTOTOXICITY STUDIES**

Cytotoxicity may be exemplified by the discovery that palitaxel inhibited mitosis by stabilising microtubules and so preventing their depolymerisation back to tubulin, in contrast to many other anticancer agents which inhibit the formation of microtubules in the first place (Cragg and Newman, 2006). The MTT assay for cytotoxicity provides a simple method for determination of live cell number in order to assess rate of cell proliferation and to screen cytotoxic agents. MTT assay measures cell viability based on the activity of mitochondria enzymes in living cells that reduce MTT to water-insoluble formazan crystals that can be easily solubilized by DMSO.

In the present study antiproliferative effect of anthocyanin extracted from *C.hirsutus* fruit on HEp-2 and MCF-7 cell lines were analyzed by conducting MTT assay. Cultures of HEp-2 and MCF-7 cell were treated with the different concentrations ranging from 7.8 -1000 μg/ml and the cell viability was counted. Control assay was carried out for sample containing only the approximately volume of blank solution and those showed no effect on cell growth (PLATE 10A and 11A). After treatment with 1000μg of anthocyanin on both the cell lines, the cells become irregular in shape and size and cell viability decrease at this concentration.
(PLATE 10B and 11B). After treatment with 125 µg of anthocyanin on both the cell line, the cells become spherical in shape and size with alter nuclear cytoplasm ratio (PLATE 10C and 11C). These have indicated that anthocyanin render some changes on the cell surface associated with the adherence of the substratum. Most of the cells had relatively flat tended appearance with long multiple cytoplasmic processes forming cross bridges with neighboring cells (PLATE 10D, 10E, 11D and 11E).

Blueberries, black chokeberries, lingonberries, and raspberries extracts were shown to decrease the proliferation of human colon HT-29 and breast MCF-7 cancer cells in a dose-dependent manner (Olsson et al., 2004). Similarly, whole cranberry purified fruit extracts were assayed for tumor growth inhibition using seven tumor cell lines and selective inhibition of K562 leukemia, and HT-29 colon cells were observed from a methanolic extract in the range IC₅₀ 16–125µg/ml (Xiaojun et al., 2002), as compared to the crude extract that only showed 50% growth inhibition at concentrations >500 µg/ml in all cell lines (H460, ME180, DU145, MCF-7, HT-29, PC3, and K562) (Xiaojun et al., 2002). The crude acidified methanol extract of C.hirsutus fruits showed results of similar to purified cranberries extract and approximately 5 fold high activities than crude cranberries extract. Thus, significant cytotoxicity is evident in the fruit extract of C.hirsutus.

The acidified methanol fruit extract of cranberries were assayed for tumour growth inhibition using eight cell lines (MDA-MB-435, MCF-7, HT-29, DU145, LNCaP, SK-MEL-5, U87 and DMS114) and identified to inhibit proliferation of all the cell lines in a dose-dependent manner (Ferguson et al., 2004). Similar results were observed in HEp-2 and MCF-7 cell lines in Cocculus hirsutus fruit anthocyanin.

The isolated polyphenols from strawberry including anthocyanins, kaempferol, quercetin, esters of coumaric acid and ellagic acid, were shown to inhibit the growth of human oral (KB, CAL-27), breast (MCF-7), colon (HT-29, HCT-116), and prostate (LNCaP,
DU-145) tumor cell line (Zhang et al., 2008). Similar results were observed in Hep2 and MCF-7 cell lines in Cocculus hirsutus fruit anthocyanin.

Anthocyanin extracted from big onion and red onion peel showed approximately 92% and 79% inhibition on HEp-2 cells and 78% and 70% inhibition on MCF-7 cells at 1000 μg/ml respectively (Geetha et al., 2012). When compared to C. hirsutus fruit anthocyanin, the big onion peel showed the significant inhibition on Hep-2 cells and lower inhibition on MCF-7 cells.

Bhavna Marya et al. (2011) studied the effects of aqueous fruit extracts on Grewia asiatica on HEp-2 and MCF-7 cell lines. The estimated IC$_{50}$ of Grewia asiatica fruit extract on Hep-2 and MCF-7 cell lines were 50.31μg/ml and 58.65μg/ml respectively. In this study the identified IC$_{50}$ value of C. hirsutus fruit extract (acidified methanol) both on Hep-2 and MCF-7 cell lines was 125 μg/ml. Grewia asiatica fruit extracts IC$_{50}$ values was approximately two fold higher than C. hirsutus fruit extract.

**CONCLUSION**

Tissue culture technology is a sole source for the conservation of germplasm and mass propagation of medicinally important plant resources like Cocculus hirsutus (L.) Diels. By standardization of the protocol for in vitro propagation of C. hirsutus will lead to identification of raw materials for comparative screening of secondary metabolites for in vivo and in vitro grown plants. The methanol and water leaf (in vitro) extracts and acidified methanolic fruit extract possessed significant biological properties and can prevent or slowing the oxidative stress related degenerative disease. The methanol extract also exhibits significant scavenging ability against different free radicals, which in turn implies hat the extract may contain active compounds which have effective antioxidant and antimicrobial activities. Hence, this species have to be explored and conserved to serve the mankind.