CHAPTER – V

DISCUSSION

5.1. EXTRACTION METHODS OF ESSENTIAL OILS

The yield of EOs is influenced by the period of collection, dehydration procedure, storage conditions of plant material and extraction methods (Pandey, et al., 2010), in addition to certain intrinsic factors like genetic profile and physiological conditions and extrinsic factors (environmental) like light, temperature, water and nutrients (Lawrence, 1986).

Commercially, EOs are extracted by three important methods which include 1) Hydro-distillation or steam distillation 2) Expression and 3) Dry-distillation. Hydro-distillation is most common used method for EO extraction; expression is used to extract EOs from citrus peel and dry distillation is used in some special and rare cases like cade oil from the wood of Juniperus oxycedrus (Baser and Buchbauer 2015; Jeyaratnam et al., 2016). In the present study EOs are extracted by using hydro-distillation method using Clevenger-type apparatus.

5.2. DURATION OF EXTRACTION

The general duration of oil extraction by using hydro-distillation varies from 2 hrs., (de-Oliveira et al., 2014), 3 hrs., (Victorio et al., 2009) and 4 hrs., (Sefidkon et al., 2004). Preliminary studies in our lab suggest that oil yield significantly increases between 3½ hrs and 3 hrs., beyond which (>3½ hrs.) there is no further increase in the total yield. According to Baser and Buchbauer (2015), increased temperature significantly reduces the distillation time. However, certain oil compounds like esters being temperature sensitive may be hydrolysed (Powar et al., 2013). In view of the above, 3½ hours under controlled temperature was considered ideal, for maximizing the yield.

Yield was generally positively correlated with nutrient level at lower temperatures, however, negatively at higher temperature (Silva et al., 2003). In the present study EO was extracted from test plants during winter seasons. Due to paucity of available material in winter, oil from Z. officinale was extracted in summer.
5.3. YIELD OF ESSENTIAL OILS

Verma et al., (2014) have reported that the yield of EO from A. marmelos of Uttarakhand ranges from 0.37 to 0.8%. In the present study the same species yielded 0.64% of EO. Low temperatures of Uttarakhand (one of the high elevation zones of India) may have positively influence the yield. Chanthaphon et al., (2008) have reported 0.57% of oil yield from C. aurantifolia of Thailand, while the yield in the present study is 0.42%. The observed variations can be attributed to geographical variations and other environmental parameters. Sarrou et al., (2013) have made an interesting observation that the age of the plant material influences oil yield, where they have reported 0.27% in young leaves and 0.45% in older leaves of plant species of Greece. The present study too older leaves of C. aurantifolia yielded 0.4% of EO.

Generally peels of citrus fruits are used for essential extraction. It is interesting to note that the leaves of C. maxima yielded (0.35%) EO on par with the peel. For example Chanthaphon et al., (2008) reported 0.24% EO yield from fruit peel of C. maxima in plants growing in Thailand. Among the rutaceae members L. acidissima yielded 0.16% EO, contrary to reports of Pande et al., (2010) who reported 0.4% in the same species.

Among the lamiaceae members, EO yield of H. suaveolens was 0.1%; this result was in agreement with earlier study of Peerzada (1997) who has also reported 0.1% from plants of Australia. While the yield of EO from M. piperita was 0.1% in the present study, Khorasaninejad et al., (2011) have reported yield ranging from 0.26 to 0.6% from the plant species of Iran.

O. basilicum yielded 0.36% of EO in the present study, while Hussain et al., (2008) reported a yield range from 0.5 to 0.8% from plants of Pakistan. O. gratissimum oil yield was 1.0%; and is in agreement with earlier study of Matasyoh et al., (2008) who reported a yield range of 0.12 to 1.4 % from plants of Kenya. However EO yield from O. sanctum was 0.2%; Sharma et al., (2014) reported a yield range of 0.8 to 1.3 % from O. sanctum.
From the foregone, it is evident that the members of lamiaceae growing in different geographical locations vary considerably in the quantity of EO yield. This variation in yield is less prominent in the members of myrtaceae (Tree species), growing in different geographical locations. It is therefore possible that herbs and shrubs (lamiaceae) are more specific in their needs to survive in an environment.

For example among three tree species of myrtaceae, *C. viminalis* yielded a range of 0.3 to 0.42% EO from the plants grown in the Brazil is on par with the present study (0.52%) (de-Oliveira et al., 2014). Similarly *E. camaldulensis* has yielded 0.67% of EO, was on par with the plants growing in Taiwan (0.67%) Cheng et al., (2009). In addition *M. linariifolia* in the present study and same species of Brazil yielded similar quantities of EOs 1.16% and 1.4% respectively (Silva, 2010).

Among the three test plants of zingiberaceae, *Alpinia speciosa* was yielded 0.58% of EO; this result in agreement with earlier study of Victorio et al., (2009) who reported yield of 0.44% from the plants of Brazil. *Z. officinale* yielded 9 times more oil (0.24%) in the present study compared to their counterparts grow in Malaysia (Sivasothy et al., 2011). Bahl et al., (2014) reported that a range of 0.05 to 0.75% EO yields from *C. longa* leaf material and similarly the same species in the present study yielded in the above range (0.56%).

*C. flexuosus* of poaceae growing in Surat, India., produced 4.5 times more EO (1.04%) compared to the test plants of the present study (0.24%) (Parikh and Desai, 2011). EO from *T. erecta* of asteraceae, yielded 0.12% and this was found to be low when compared to the previous results of Sefidkon et al. (2004) who reported 0.37% from plants of Iran.

In the present study that highest yield of EO was observed in plants of myrtaceae (ranges from 0.52 to 1.16%) followed by zingiberaceae (ranges from 0.58 to 0.24%), rutaceae (ranging from 0.16 to 0.64%), lamiaceae (ranging from 0.1 to 0.36%), poaceae (0.20 %) and asteraceae (0.12%). Amongst 18 test plants, highest EO yield was observed with *M. linariifolia* (1.16%) and lowest was with *H. suaveolens* and *M. piperita* (0.1% each) of lamiaceae. However, there is no direct coordination between the quantity of EO produced and biological activity.
Thus as rightly pointed out by Lawrence, (1986), EO yield is influenced by genetic profile, physiological conditions and environmental conditions such as light, temperature, water.

5.4. ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS

All test EOs possessed antimicrobial activity. However the extant of activity against the test organisms is considerably variable. The relative antimicrobial efficacy of oils is in the order of *C. albicans* (Fungi) > Gram-positive bacteria > Gram-negative bacteria. Many oils of rutaceae have showed potential antimicrobial activity followed by zingiberaceae, lamiaceae and myrtaceae oils; antimicrobial activity of *C. flexuosus* (poaceae) EO is on par with EOs *C. aurantifolia* and *C. maxima* of rutaceae. Relatively low antibacterial activity was observed in *T. erecta* (asteraceae); nevertheless it showed potential anticanidal activity. All the test organisms found to be sensitive in the entire study to the EO of *A. speciosa* only. To the best of our knowledge this work is the first attempt ever made to give comprehensive comparison of antimicrobial activities of EOs and their respective compound/s from plants belongs to a wide range of families.

5.4.1. Antimicrobial activity of rutaceae essential oils

Singh *et al.*, (1993) have identified 1,8-cineole as one of the key constituent (27.15%) and also noticeable amount of α and β-pinene in *A. marmelos* EO. Garg *et al.*, (1995) have reported α-pinene (0.72%) and β-pinene (1.05%) in the leaf EO of *A. marmelos*. Similar studies have also reported pinene as a major continent of *A. marmelos* EO (Satyal *et al.*, 2012; Ibrahim, 2015). Contrary to the above observation in the present study two antimicrobial compounds viz. 2-hydroxycineole (derivative of 1,8-cineole) and pinanediol (ethylene glycol ether of pinene) were identified.

Ekade and Manik, (2014) and Mujeeb *et al.*, (2014) have reported that pinanediol has antimicrobial activity. Miyazawa and Hashimoto (2002) and Knight, (2009) have reported the antimicrobial activity of 2-hydroxycineole. TLC-bioautography in the present study endorsed the antimicrobial efficacy of 2-hydroxycineole and pinanediol.
In accordance with the present study, Mujeeb et al., 2014 have reported 0.97% pinanediol in the methanolic extract of A. marmelos leaf. According to Gozhina (2013) pinanediol is a more effective antimicrobial than pinene. Hence, in order to enhance the biological activity, pinene was converted to pinanediol to synthesize pinanediol borates. Thus A. marmelos leaf material used in the present study is a more potent antimicrobial due to pinanediol and 2-hydroxycineole. In the present study the sensitivity of S. epidermidis, A. baumannii and C. albicans to the EO of A. marmelos was on par with positive controls Gentamicin and Ketoconazole respectively.

Among the 8 major EO components of C. aurantifolia, five antimicrobial compounds Z and E-citral (neral and geranial), cis-geraniol, geraniol and geranyl acetate, were identified as antimicrobials. All these compounds are citral isomers or its derivatives. Selvaraj et al., (2002) have reported the presence of 24% of neral, 33% of geranial and 2.1% of geranyl acetate in C. aurantifolia EO, while Lota et al., (2002) have reported the presence of 0.1% of geraniol in the leaf EO of C. aurantifolia along with other compounds.

Dorman and Deans (2000) have also reported the potential antimicrobial activity of Z and E-citra, cis-geraniol, geraniol and geranyl acetate. As evident in the Tables 17 & 28, C. aurantifolia EO alone (containing citral, its derivatives and its isomers) is more effective antimicrobial than citral alone, suggesting that there is an interaction between these compounds lead to an additive effect of the oil.

C. aurantifolia is found to be highly effective against 10 of the 17 test organisms studied presently. The EO is almost two times more effective than Gentamicin in some organisms like B. cereus, B. subtilis, S. aureus, A. baumannii, P. mirabilis, V. cholerae and C. albicans.

EO of C. aurantifolia peel contains almost similar components as that of leaf. Thus far, commercial EO is solely being produced from fruit peels and not leaves. Thus use of C. aurantifolia leaves for commercial production EO is also seems to be a viable preposition.
Among the 8 major constituents identified in EO of *C. maxima* only five compounds such as Z and E-citral, cis-geraniol (Z-nerol), geraniol (E-nerol), methyl cinnamate, linalool and eucalyptol are identified as antimicrobial compounds. Earlier studies have also reported the presence of all these compounds in the leaf EO of *C. maxima* (Singh *et al.*, 2010; Varkey *et al.*, 2014).

A similar antimicrobial activity was observed in the combinatorial study with citral, linalool and eucalyptol combination and EO of *C. maxima*, particularly against *E. coli*, *K. pneumoniae* and *S. marcescens* (gram-negative bacteria). Conversely oil was found to be more active than its corresponding tested combination against many test organisms including *C. albicans* (Figure 26). The observed variations in the antimicrobial activity spectrum can be attributed to the other antimicrobial compounds detected in the *C. maxima* EO viz. methyl cinnamate and linalool.

The composition of EO of *C. aurantifolia* and *C. maxima* is very similar and it is not surprising that they share a strikingly similar antimicrobial activity. *C. maxima* differ from *C. aurantifolia* in the presence of eucalyptol, linalool and methyl cinnamate and furthermore presence of geranial acetate in *C. aurantifolia*. This similarity in composition yielded almost similar zone of inhibitions, however relatively high zone of inhibitions were observed with *C. maxima* EO especially against gram-negative bacteria and yeast such as *S. pyogenes*, *A. baumannii*, *S. enterica ser. typhi*, *S. flexneri*, *V. cholerae* and *C. albicans*.

Elaissi *et al.*, (2012) have reported that eucalyptol enhances the absorption of other compounds of the oil through the membranes of the microorganisms and thereby, enhancing the antimicrobial activity of the EO. This appears true particularly to the gram-positive bacteria in the present study. For example activity citral and linalool combination was improved greatly after addition of eucalyptol (citral, linalool and eucalyptol combination), particularly against gram-positive bacteria (Table 29). However, the presence of eucalyptol has not enhanced the activity of oil against many gram-negative bacteria.

*C. maxima* leaf EO is not having commercial significance with in India and globally. However, a close similarity between the antimicrobial activity of *C. maxima* and *C.
*aurantifolia* supports that *C. maxima* can be commercially exploited for EO production.

Out of 11 major constituents of *C. aurantium*, only eucalyptol, linalool, L-terpinen-4-ol and α-terpineol were identified with bioautography. In accordance with the present study Huang et al., (2000) have reported that the presence of 0.5% of 1,8-Cineole, 10% of linalool, 0.3% of terpinen-4-ol and trace amounts of α-Terpineol.

Bacteria such as *S. pyogenes*, *A. baumannii*, *E. coli* and *V. cholerae* are found to be very sensitive to the EO of *C. aurantium* compared to positive control Gentamicin. Very interestingly, activity of this oil against *V. cholerae* is so significant; the interactions of eucalyptol, linalool, L-terpinen-4-ol and α-terpineol could have enhanced the activity of this oil.

In the essential of *L. acidissima* 13 major EO constituents were identified with TLC-bioautography viz. eugenol, L-terpinen-4-ol and α-terpineol. Syamasundar et al., (2010) has reported that 0.6 to 1.5% of terpinen-4-ol, 1to 1.2% α-terpineol and significant amounts of eugenol derivatives present in the EO of *L. acidissima* plant native to the western ghats, India. Senthilkumar et al., (2013) also reported that 6.5% of eugenol in the EO of *L. acidissima*.

L-terpinen-4-ol and α-Terpineol are common in *C. aurantium* and *L. acidissima* EOs. Further, addition of eugenol in *L. acidissima* does make it an effective antimicrobial than *C. aurantium* especially against *P. mirabilis*. L-terpinen-4-ol, α-terpineol and eugenol are therefore found to be synergistic and yielded additive effect against *P. mirabilis*. Conversely absence of eugenol makes *C. aurantium* a week antimicrobial than *L. acidissima*.

As already mentioned, citral is found to be a potential antimicrobial and the absence of citral in the three species of rutaceae viz. *A. marmelos*, *C. aurantium* and *L. acidissima* makes these EOs week antimicrobials.

**5.4.2. Antimicrobial activity lamiaceae essential oils**

*H. suaveolens* EO was composed of 10 major constituents and amongst them only three compounds 1,8-Cineole, L-terpinen-4-ol and α-trans-bergamotenol were
identified by TLC-bioautography. Hitherto many researchers have confirmed the presence of 1,8-cineole, L-terpinen-4-ol and α-trans-bergamotanol in EO of *H. suaveolens* found in various regions of the world (Mallavarapu *et al*., 1993; Peerzada, 1997). However none of these authors have attributed antimicrobial activity to α-trans-bergamotanol. Hence further studies are much needed for confirmation of α-trans-bergamotanol as an antimicrobial compound. On the other hand it is also presumed that the RF values of α-trans-bergamotanol is very close to the other compounds identified in the zone of inhibition on TLC plate of bioautography.

As evident in the Table 18, six test organisms have not shown any visible inhibition zones and rest have showed zone of inhibition less than 13.7±0.58 mm with EO of *H. suaveolens*. This clearly signifies the low antimicrobial efficacy of *H. suaveolens* EO. It was also evident that the presence of eucalyptol does not influence the overall antimicrobial efficacy of EO.

Out of 29 major constituents of *M. piperita*, only four different compounds eugenol, linalool and methyleugenol, mint furanone were identified in TLC-bioautography. The presence of linalool and mint furanone in *M. piperita* EO was previously reported by Lawrence (1993) and Iscan *et al*., (2002). However, none of them have reported the presence of eugenol and methyleugenol in *M. piperita* EO.

The antimicrobial activity of this oil is on par to more potential compared to the positive control Gentamicin against *B. subtilis*, *A. baumannii* and *V. cholerae*. Zone of inhibition shown by these bacteria are comparable or even more than positive control used in this study. Furthermore amongst all the 18 test EO *M. piperita* is found to possess highest anticandidal activity.

Souza *et al*., (2005) have reported potential antifungal efficacy of eugenol against *Aspergillus* spp., *Penicillium* spp., and *Rhizopus* spp. The presence of mint furanone in *M. piperita* has been confirmed by previous researchers but none of them have attributed the antimicrobial activity to the compound (Lawrence, 1993; Iscan *et al*., 2002). Hence this compound has to be further elucidated to confirm its antimicrobial property.
Apart from the *M. piperita*, presence of eugenol and linalool combination was also seen in other lamiaceae test oils such as *O. basilicum* and *O. sanctum*. This similarity yielded almost similar zone of inhibitions with majority of test organisms (Figures 28, 29 & 30). A prominent comparable activity of eugenol and linalool combination and *M. piperita* EO was observed (except *B. subtilis*, *P. mirabilis*, *A. baumannii*, and *C. albicans*). However, as evident in the Figure 28, oil was found to be active against *B. subtilis* and *C. albicans*, conversely linalool and eugenol combination was active against *P. mirabilis*.

Among the 6 major constituents three compounds eugenol, linalool and methyl cinnamate were identified by TLC-bioautography in the *O. basilicum* EO. Politeo et al., (2007) have reported that 5.9% of eugenol, 28.6% of linalool and 1.6% of methyl cinnamate in the EO of *O. basilicum* species from Croatia. Similarly Vina and Murillo (2003) have reported that 4.1% of eugenol, 13.33 to 33.03% of linalool and 2 to 9.5% of methyl cinnamate in the various chemotypes of *O. basilicum* from Colombia.

Sensitivity of *B. subtilis*, *S. pyogenes*, *A. baumannii*, *V. cholerae* and *C. albicans* to the *O. basilicum* EO was comparable with its respective positive control Gentamicin. In addition the oil was more potential antifungal than its corresponding positive control Ketoconazole. Interestingly *S. pyogenes* showed highest sensitivity when compared to the oils having almost similar compounds (eugenol and linalool) such as *M. piperita* and *O. sanctum*. May be the presence of methyl cinnamate made *O. basilicum* oil a stronger antimicrobial against *S. pyogenes*.

Gupta (2011) has reported the antimicrobial activity of eugenol and linalool oxide, tested by broth dilution method against *P. aeruginosa*, *E. coli*, *S. aureus* and *C. albicans*. Pasqua et al., (2007) reported that methyl cinnamate in combination with eugenol inhibited the growth of some human pathogenic bacteria, Stefanovic et al., (2015) reported that methyl trans-cinnamate alone has antibacterial and antifungal properties. Hence it is possible that eugenol, linalool and methyl cinnamate collectively contributed for the antimicrobial activity of *O. basilicum*.

Out of 10 major compounds, four antimicrobial compounds were identified viz. α-cadinol, eugenol, m-eugenol, Z-citral in the *O. gratissimum* EO by TLC-
bioautography. Presence of these compounds was also reported by many researchers across the globe (Bhattacharya et al., 1996; Dambolena et al., 2010; Owokotomo et al., 2012).

The antimicrobial activity of *O. gratissimum* oil was comparable to the positive control Gentamicin against *B. subtilis*, *S. epidermidis*, *A. baumannii*, *P. mirabilis*, *S. flexneri* and *V. cholerae*. As evident in the Figure 27, the antimicrobial activity of citral and eugenol combination and *O. gratissimum* EO alone are strikingly very close. However, the interaction of other compounds (m-eugenol and α-cadinol) many improve oil activity than citral and eugenol combination, particularly against *S. flexneri*, *V. cholerae* and *C. albicans*.

Reports on direct testing of antimicrobial activity of m-eugenol not available in the literature, nevertheless Sugumaran et al., (2011) and Vyry et al., (2014) have attributed antimicrobial activity to m-eugenol along with other compounds of *Piper betle* and *Ocimum canum* oils respectively. Ho et al., (2010) have tested and reported antimicrobial activity of standard α-cadinol by broth dilution method against many similar test organisms of the present study.

Unlike other lamiaceae oils, *O. gratissimum* oil is a potential antimicrobial against *S. flexneri* and *P. mirabilis*. As evident in the Table 17, the sensitivity of these two organisms was strikingly similar to the other citral containing EOs of the study viz., *C. aurantifolia*, *C. maxima* and *C. flexuosus*. Hence it was probable that the presence of citral makes this oil more antimicrobial than other EOs of lamiaceae.

Among 13 major compounds, only four compounds eugenol, linalool, m-eugenol, methyleugenol were identified in the *O. sanctum* EO with TLC-bioautography. Sims et al., (2014) has reported that 0.1 to 0.7% of Linalool, 25.3 to 51.5% of eugenol and notable amount of methyleugenol was available in the different chemotypes of *Ocimum tenuiflorum* (formerly classified as *O. sanctum*) of America. Previous reports of Dorman and Deans (2000); Sugumaran et al., (2011) and Vyry et al., (2014) suggest the antimicrobial activity of citral, linalool and m-eugenol against many bacteria and fungi.
The antibacterial activity *O. sanctum* is comparable with positive control Gentamicin against *A. baumannii, V. cholerae*. The activity of eugenol and linalool combination is also comparable with *O. sanctum* EO against most test organisms, except *K. pneumoniae, P. mirabilis* and *C. albicans* and Particularly *C. albicans* was more sensitive to the tested combination than the *O. sanctum* EO. Standard eugenol tested in this study showed significant antibacterial activity against certain gram-positive and gram-negative bacteria and potential anti conidial activity. Hence, the antimicrobial activity of *O. sanctum* can be attributed to the compounds identified in TLC-bioautography.

5.4.3. Antimicrobial activity myrtaceae essential oils

Out of 10 major constituents of *C. viminalis*, five compounds viz. eucalyptol, linalool, L-terpinen-4-ol, L-α-terpineol and α-terpineol were identified with TLC-bioautography. Except linalool and eucalyptol, rest of the three compounds are isomers. The presence these compounds was also reported in the EO extracted from similar species from South Africa by Oyedeji et al., (2009)

The antibacterial activity of this *C. viminalis* EO is comparable with positive control Gentamicin particularly against *S. aureus, S. epidermidis, S. pyogenes, A. baumannii* and *P. mirabilis*. Anticandidal activity of this essential is also found to be comparable with its respective positive control Ketoconazole. The interaction of isomers of three terpineols (L-terpinen-4-ol, L-α-terpineol and α-terpineol) with linalool and eucalyptol make this oil potential, particularly against above mentioned test organisms.

This is the first report to the best of our knowledge to point out the similarity of EOs of myrtaceae (*C. viminalis*) and zingiberaceae (*A. speciosa*) members with reference to the presence of L-terpinen-4-ol, L-α-terpineol, α-terpineol, eucalyptol and linalool. Although *C. viminalis* and *A. speciosa* share many components, EO of *A. speciosa* is the only test oil found to be potentially inhibited the growth of all the 17 test organisms in the present study. It is assumed that methyl cinnamate and myristic acid present in EO of *A. speciosa* enhances the antimicrobial activity of the oil.
Among 14 major compounds five compounds were detected in bioautography (eucalyptol, β-eudesmol, γ-eudesmol, methyl cinnamate and piperitone) in the *E. camaldulensis* EO. The reports of Traore *et al.*, (2014) have confirmed the presence of 19.9 to 90.4% of eucalyptol; 0.1 to 0.2% of γ-eudesmol and 0.1 to 0.3 % of piperitone in the EO of *E. camaldulensis* from Mali. The antibacterial activity of *E. camaldulensis* EO is on par or more active than the positive control Gentamicin against *S. aureus*, *S. epidermidis*, *S. pyogenes*, *A. baumannii*, *P. mirabilis* and *V. cholerae*. Anticandidal activity is also compared to its corresponding positive control Ketoconazole.

Amongst 18 test oils, both the isomers of eudesmol (β -eudesmol, γ-eudesmol) are detected only in *E. camaldulensis* EO. Costa *et al.*, (2008) have quoted that β-eudesmol, γ-eudesmol contribute for many biological activities including antimicrobial activity. Stefanovic *et al.*, (2015) have reported antimicrobial activity of methyl cinnamate against certain gram-positive and gram-negative bacteria. Sokmen *et al.*, (2004) have reported that piperitone possess antimicrobial activity. Hence it was probable that interaction all the five compounds are collectively responsible for antimicrobial activity of *E. camaldulensis* EO.

Out of 17 major constituents, only four compounds were identified with TLC-bioautography viz., cubenol, eucalyptol, myristic acid and α-terpineol from *M. linariifolia* EO. Padalia *et al.*, (2015) have reported that 77.4% of eucalyptol, 7.72% of α-terpineol and 0.25% of 1-epi-cubenol (stereoisomer of cubenol) were present in *M. linariifolia* oil. The antibacterial activity of *M. linariifolia* was comparable with Gentamicin against *A. baumannii*, *S. flexneri* and *V. cholerae*.

Solis *et al.*, (2004) have reported that cubenol possess highest antimicrobial activity amongst all the sesquiterpenes. Thormar (2011) has reported that unsaturated fatty acids like myristic acid possess antimicrobial activity and Huang *et al.*, (2010) also opined the same. Very interestingly Liu and Huang, (2012) have reported that myristic acid enhances the absorption of curcumin and thereby enhanced the antimicrobial activity of curcumin. Therefore it presumed that antimicrobial activity of *M. linariifolia* EO is due to the presence of identified compounds by bioautographic studies.
5.4.4. Antimicrobial activity zingiberaceae essential oils

Out of 13 major compounds identified in A. speciosa a highest of seven compounds (eucalyptol, linalool, methyl cinnamate, myristic acid, α-terpineol, its stereoisomer L-α-terpineol and L-terpinen-4-ol) were detected by TLC-bioautography. Presences of these compounds have been confirmed by the previous studies of Upadhyay et al., (2014) from Alpinia zerumbet (Syn. A. speciosa) EOs.

A worthy observation made in the present study is that E. faecalis is known to be resistant to many test oils, is susceptible to the essential of A. speciosa. It is equally interesting to note that EO of C. viminalis possessing almost same components.

Though the EO of A. speciosa does not possess citral or eugenol (potential antimicrobials in the study), its activity is found to be unmatched with the rest of test oils. It is probable that the interaction of all the compounds together with absorption enhancement by eucalyptol and myristic acid through the membranes may makes this oil potentially antimicrobial (Elaissi et al., 2012; Liu and Huang, 2012).

Four compounds eucalyptol, hydroxy-1-8-cineole, linalool and α-terpineol have been detected in the C. longa EO by bioautographic studies. Presence of these compounds in the leaf EO of C. longa was also reported by Srivastava et al., (2005). As only two organisms, A. baumannii and P. mirabilis are sensitive to EO, it is presumed that α-terpineol alone with combination with the other compounds does not contribute much for the antimicrobial activity of this oil.

A highest of seven compounds; borneol, cartol, Z and E- citral, eugenol, meta-eugenol and linalool were identified with TLC-bioautography in Z. officinale EO. The presence of these compounds was also reported from leaf EO of Z. officinale by Sivasothy et al., (2011) and Bellik (2014).

The antimicrobial activity this oil was on par to more potential than its corresponding positive control Gentamicin against B. subtilis, S. epidermidis, A. pyogenes, A. baumannii, P. mirabilis and S. marcescens. The antimicrobial activity of this EO is almost comparable to the other citral congaing essentials such as C. aurantifolia, C.
maxima, C. flexuosus. As this EO possesses citral and eugenol, the activity is also comparable with other similar compounds containing EO (O. gratissimum). As evident in the Figure 25, the activity of this Z. officinale oil almost comparable to citral, linalool and eugenol combination tested in this study particularly in gram-positive bacteria and C. albicans. Oil was more active than studied combination against many gram-negative test bacteria. It may be probable that methyl cinnamate, borneol and cartol reasoned for the difference.

5.4.5. Antimicrobial activity C. flexuosus and T. erecta essential oils

Five antimicrobial compounds viz. Z and E-citral, geraniol, cis-geraniol and (S)-cis-verbenol were identified in C. flexuosus EO. Reports of Mohammad et al., (1981) have confirmed the presence of 33.1% of neral (Z-citral), 48.15% of geranial (E-citral), 1.4% of geraniol and 0.8% of cis-geraniol (nerol) in the C. flexuosus EO. This oil had similar antimicrobial compounds as that of C. aurantifolia and C. maxima of rutaceae and thus, it was not surprising that these three oils possessed similar antimicrobial activity against majority of test organisms.

Five antimicrobial compounds eucalyptol, linalool, L-terpinen-4-ol, (S)-cis-verbenol and piperitone were identified in T. erecta EO. Machado et al., (1994) have confirmed the presence of all these compounds in the leaf EO of T. erecta except (S)-cis-verbenol.

Antibacterial activity of EO of T. erecta is on par or more with positive control especially against A. baumannii and V. cholerae. The EO of T. erecta and C. viminalis are very close in their antimicrobial components. However T. erecta possess high antimicrobial activity. These differences can be attributed to the quantity compounds present in the oil. Thus it is evident that quantity of compounds is also a determinating factor contributing oil’s efficacy.

5.5. SENSITIVITY OF TEST ORGANISMS TO ESSENTIAL OILS

Generally Gram-negative bacteria are more resistant to EOs than Gram-positive bacteria (Nazzaro et al., 2013). Similarly this phenomenon is proved right in majority
of test oils against many test bacteria in the present study. Nazzaro et al., 2013, has rightly pointed out that Gram-positive bacterial cell wall allows the penetration of hydrophobic molecules easily. Conversely, Gram-negative bacteria cell wall is more complex and this complexity makes Gram-negative bacteria more resistant to EOs (Nazzaro et al., 2013).

Amazingly, among all the test bacterial strains, A. baumannii (Gram-negative) is found to be highly sensitive to all the test EOs. Contrary to this E. faecalis (Gram-positive) was found to be highly resistant. As E. faecalis is a capsulated bacteria it is presumed that the capsule may prevent penetration of EO components and hence many EOs do not have any activity with this organism. However the EO of A. speciosa is known to have an effect on E. faecalis. Whether the oil components of A. speciosa have any effect on the capsule needs to be further investigated.

5.5.1. Sensitivity of Gram-positive bacteria

The food borne pathogenic strain B. cereus is found to be more sensitive with C. aurantifolia, C. maxima, A. speciosa, Z. officinale and C. flexuosus. Interestingly all the oils are known to possess citral as key constituent.

A pathogenic strain S. aureus is found to be very sensitive to C. aurantifolia, C. maxima, E. camaldulensis, A. speciosa, and C. flexuosus. It seems that S. aureus is sensitive to oils with citral combinations, and very interestingly oil without citral (E. camaldulensis) is also effective against S. aureus.

O. gratissimum and Z. officinale are also citral containing EOs but they are not as much potential as other citral containing EOs. It may be plausible that the amount of citral is insufficient to elicit the activity on par with other citral containing EOs or one of the compounds in the oil antagonising the oils activity particularly against S. aureus. As evident in the Table 29, citral alone and in-combination with other tested combination also showed potential activity against S. aureus in the present study.

A clinical isolate (isolated from skin lesion) S. epidermidis is found to be very sensitive to A. marmelos, C. aurantifolia, C. maxima, O. gratissimum, E.
camaldulensis, A. speciosa, Z. officinale and C. flexuosus. Except A. marmelos and E. camaldulensis rest of the oils are having citral and as a key EO constituent. Similarly as evident in the Table 29, citral alone and in combination with other compounds showed potential antibacterial activity against S. epidermidis.

S. pyogenes is a pathogenic strain, found to be sensitive with C. aurantifolia, C. aurantium, C. maxima, C. viminalis, A. speciosa, Z. officinale and C. flexuosus. Among above test oils four oils contain citral as their key constituents; similarly as evident in the table 29, citral alone and in combination showed potential antibacterial activity against S. pyogenes. Rest of the oils possess isomers of terpineol (L-terpinen-4-ol, α-terpineol and L-α-terpineol) in toto or partially. Hence it is probable that these compounds also possess potential antibacterial property against S. pyogenes.

5.5.2. Sensitivity of Sensitivity of Gram-negative bacteria

P. mirabilis is highly sensitive with C. aurantifolia, L. acidissima, C. maxima, O. gratissimum, C. viminalis, E. camaldulensis, A. speciosa, C. longa, Z. officinale and C. flexuosus. All the five citral containing EOs showed potential activity and similarly citral and its combinations have also showed high antimicrobial activity in the present study. As evident in the Tables 28 & 29, citral and eugenol combination antagonising each other, similarly as evident in the Tables 18 & 20, activity was slightly depleted in citral and eugenol containing EOs (O. gratissimum and Z. officinale) against P. mirabilis. A. speciosa, C. viminalis, C. longa are having isomers terpineols (L-terpinen-4-ol, α-terpineol and L-α-terpineol) in toto or partially. Hence it is probable that these compounds possess potential antibacterial property against P. mirabilis.

Typhoid fever causing bacteria S. enterica. Ser. typhi showed potential sensitivity to C. aurantifolia, C. maxima, A. speciosa, Z. officinale EOs. Conversely very low activity was observed C. flexuosus (compositionally similar with C. maxima and C. aurantifolia). Here it may be possible that (S)-cis-verbenol present in C. flexuosus antagonises the activity of citral and its isomers against S. enterica. Ser. typhi.

Dysentery causing pathogenic bacteria S. flexneri is found to be very sensitive to C. aurantifolia, C. maxima, O. gratissimum, C. viminalis, M. linariifolia, A. speciosa,
Z. officinale and C. flexuosus. All the five citral containing EOs have shown potential activity, similarly citral alone and with combination of other compounds also showed potential activity in the present study. Although citral is absent in C. viminalis, M. linariifolia, A. speciosa, they possess some or total isomers of terpineols (L-terpinen-4-ol, α-terpineol and L-α-terpineol) as their key constituents in EO. Hence it is probable that these compounds alone or in combination possess potential antimicrobial activity against S. flexneri.

Cholerae causing pathogenic bacteria V. cholerae, is potentially sensitive to majority of test oils except A. marmelos, L. acidissima, H. suaveolens and C. longa. All the potentially active oils are found to possess citral or eugenol with combination of other compounds. Certain EOs viz. C. viminalis, M. linariifolia, A. speciosa have isomers of terpineols. Citral and eugenol with combination of other compounds have also showed potential antibacterial activity against V. cholerae in the present study.

Among the remaining Gram-negative test bacteria none of the test oil has potent enough on par with positive control Gentamicin against E. aerogenes, P. aeruginosa and S. marcescens. A. speciosa only showed potential activity against E. coli and K. pneumoniae.

Highest anticandidal activity was observed with M. piperita EO and the lowest was with L. acidissima and H. suaveolens. It is possible that the synergistic interaction of combination of eugenol, methyleugenol, linalool and mint furanone made M. piperita EO more anticandidal than other oils.

EO from A. speciosa is so significant, though this oil doesn’t contain citral or eugenol combinations; all the test organisms found to be sensitive with this oil. Particularly interaction of isomers terpineols (L-terpinen-4-ol, α-terpineol and L-α-terpineol) made this oil potentially antimicrobial. It is also plausible that the presence of myristic acid and eucalyptol enhanced the absorption of above compounds through cell membrane and collectively influenced the activity.

5.6. COMBINATORIAL STUDIES OF COMPOUNDS

Antimicrobial activities of various compounds of EOs were studied and reported by Aridogan et al., (2002); Mesa-Arango et al., (2009); Raman et al., (1995), Souza et
al., (2005); Dorman and Deans (2000); Inouye et al., (2001) Mith et al., (2014) Vimal et al., (2013) and Stefanovic et al., (2015). Nevertheless combinatorial studies on EO compounds for their possible interactions are meagre. However, a few recent studies that need mention are those of Zhou et al., (2007); Van Vuuren and Viljoen, (2009); Mulyaningsih et al., (2010). Bassole and Juliani, (2012) have provided an excellent review on EO compounds and their interactions that lead to additive, antagonistic and synergistic effects.

Lambert et al. (2001) have reported additive effect of thymol/carvacrol on S. aureus, P. aeruginosa and E. coli. Conversely the same combination is observed to be antagonistic on S. aureus and P. aeruginosa and E. coli (Gallucci et al., 2009). In the study of Pei et al. (2009), thymol/eugenol and carvacrol/eugenol combinations were found to possess synergistic interaction against E. coli, where as Gallucci et al., (2009) have reported antagonisms with carvacrol/eugenol against E. coil, B. cereus and S. aureus.

In the present study too, eucalyptol and linalool alone were least effective antimicrobials, but their combination showed synergistic interaction against S. epidermidis, P. mirabilis and S. marcescens. Thus, the results of the present study and the available literature clearly indicates that the interactions are case specific and seem to be greatly influenced by bacterial strains used for the study.

Addition of linalool either to citral or eugenol enhanced the activity significantly. However, addition of linalool enhances eugenol’s activity more than that of citral’s.

Individually citral and eugenol are potential antimicrobials. Conversely their combination is antagonistic against many test organisms. A clear additive effect was noted against S. epidermidis, S. pyogenes, while synergistic effect was noted against P. aeruginosa.

Addition of a third compound (linalool) to the above combination (citral/eugenol) had not enhanced the antimicrobial activity significantly. A combination of linalool, citral and eugenol had significantly reduced the activity against gram-negative bacteria, suggesting that these components are antagonizing each other.
Interestingly replacement of eugenol with eucalyptol to the above combination (citral, linalool and eucalyptol) had significantly enhanced the activity against A. baumannii, E. coli, V. cholerae, S. epidermidis, P. aeruginosa and S. flexneri.

The antimicrobial efficacy of individual as well as combination of oil components have been evaluated and compared with the EOs as whole. In accordance with our hypothesis, we have identified those combinations that have additive, synergistic or antagonistic effect.

For example a combination of citral, eugenol and linalool has more antimicrobial activity than the EO of Z. officinale which containing 10 components in addition to the citral, eugenol and linalool. This suggests, that other than the three said compounds, majority of the oil components do not have a significant role in the EO’s activity.

Similarly citral, linalool and eucalyptol seem to be as effective as the EO of C. maxima possessing five major components, apart from the above said compounds, suggesting a minor role of these compounds in the oil’s antimicrobial activity.

In the present study oils of lamiaceae (O. basilicum, O. sanctum, M. piperita) possessing linalool and eugenol have a strikingly similar antimicrobial activity. In conclusion, it can be said that oils with similar components possibly have similar antimicrobial activities. This is further exemplified by the fact that oils of C. maxima and C. aurantium possessing same components had similar antimicrobial spectrum.

Thus this study has focussed on identification of combination of components of EOs with potential antimicrobial activity.

6. CONCLUSIONS

In the present study a new method was standardized for detection of bioactive compounds from EOs using TLC-bioautography.

This study is unique as it has demonstrated the possible use of commercially available TLC-Plates for isolation and identification of antimicrobial compounds from EOs.
Antimicrobial activities of many test oils were found to be on par with positive control (10 mcg Gentamicin) against majority of test organisms.

Majority of test oils have proved to be potentially effective against pathogenic microorganisms such as food borne pathogens, wound infections, typhoid fever, cholera and dysentery causing pathogens, nosocomial infections, candidias etc. Therefore formulations using these oils or their components as safe and alternative medications for antibiotics can be explored. Hence there is a need to examine the application of EOs and their components as antimicrobial agents.

To the best of our knowledge this work is the first attempt ever made to give comprehensive comparison of antimicrobial activities of EOs and their respective compound/s from plants belonging to a wide range of families.

The study has contributed a valuable data for future research to formulating these individual antimicrobial compounds into various useful products.

7. RECOMMENDATIONS

The antimicrobial activity and yield of EOs from leaves of citrus plants is on par with the fruit peels, which are being presently used commercially for EO extraction. Awareness should be created in farming community to extract EOs during non-productive seasons from leaves for additional financial benefits.

Similarly in the plants of zingiberaceae, rhizome is commercially valuable and foliage is generally ignored. Farmers can be encouraged to make use of foliage for oil extraction for additional income.

Role of EOs has been almost reduced to use in the preparation of perfumes, cosmetics and food flavourings, while their use in pharmaceutical preparations is declining drastically. Promotion of research activities on EO is much needed to explore therapeutic potentialities

As many EO components are responsible for some allergic reactions, these kind of combinatorial studies are much needed to capture the advantage of intended application of oil compound(s).
Food borne pathogens such as *B. cereus*, *S. enterica* var. *typhi* and *V. cholerae* are known to highly sensitive to *C. aurantifolia*, *C. maxima*, *M. linariifolia*, *A. speciosa*, *Z. officinale* and *C. flexuosus*. Hence, these oils can be used as preservatives to enhance shelf life of foods. Alternatively, EOs can also be used in active food packing.

*A. speciosa* is currently an endangered species. It has several similarities and better qualities than Tea tree oil (*M. linariifolia*) which is being imported in India. The promotion of cultivation of this plant is beneficial to the farmer and for health promotion.

As an opportunistic pathogenic bacterium, *A. baumannii* is becoming increasingly resistant to conventional chemotherapeutic agents. All EOs in the present study are highly effective against this nosocomial bacterium. Thus, suitably formulated EOs can be used as surface disinfectants and antiseptics in hospital and food industries. It is necessary to promote cultivation of EO producing plants species on a large scale particularly in Andhra Pradesh. This can be a significant commercial farming proposition.