DISCUSSION

Oral squamous cell carcinoma is the sixth most common neoplasm associated with high morbidity and mortality rates. Despite several recent advances in the therapeutic strategies the five-year survival rate has not seen much improvement. Additionally delivering an affordable and equitable cure for cancer is yet another challenge in a developing country, India.

With regard to the pathogenesis of oral squamous cell carcinoma, it is well known that oxidative stress and free radical mediated damage plays a pivotal role. It has also been documented that a diseased state is often characterized by overzealous production of free radicals and a jeoparadised state of antioxidants. Moreover microorganisms such as *Streptococcus mutans* and *Candida albicans* have also been implicated in carcinogenesis. Microorganisms are known to alter the cell cycle and apoptosis evasion ultimately leading to cancer. They can also metabolize procarcinogens and cause increased nitrosamine production. *Candida abicans* is known to cause chronic inflammation and can alter the tumor microenvironment favoring metastasis.

It could hence be understood that an ideal anticancer agent should work on not only bringing about changes in cell cycle and induce apoptosis but also should have antioxidant and antimicrobial properties. A therapeutic approach for cancer that would be cost effective and that would possess anticancer, antimicrobial and antioxidant properties would be most ideal agent in comparison with the current chemotherapeutic agents that causes several adverse effects such as nausea,

vomiting and immunocompromised state but do not have antioxidant and antimicrobial properties as discussed above. In this regard, herbs, fruits, vegetables have been extensively explored for their anticancer potential.

With the available literature, the present study was conducted to assess the antioxidant, antimicrobial and anticancer properties of *Trigonella foenum-graecum* L. (seeds), *Cinnamomum verum* J. Presl (bark), *Carica papaya* L. (leaves of male and female plant) and *Carica papaya* L. (seeds).

The present herbs were chosen due to their abundant availability and documented daily use in Indian cusine. Moreover India is one of the important producers of all the three herbs. To test the antioxidant potential the above mentioned herbs were subjected to Total antioxidant activity assay **(Prieto *et al*., 1999),** Reducing power assay **(Oyaizu, 1986)**, Nitric oxide scavenging activity assay **(Alderton *et al*., 2001)**, Diphenyl picryl hydrazyl scavenging activity assay (**Yokozawa *et al*., 1998**) and Superoxide radical scavenging activity assay (**Robak and Gryglewski, 1988**). Total Antioxidant capacity was used to evaluate the antioxidant activity of the plant extracts based on the reduction of Molybdenum by the sample analyte and the subsequent formation of green phosphate complex. The reducing power assay is based on the ability of the extract to reduce ferric ions and form a colored complex. The reducing capacity of a compound could be an indicator of its potential antioxidant activity. The reducing ability is usually associated with the presence of reductones, that break the free radial chain by hydrogen atom donation (**Subhashini *et al*., 2011**). Thus Reducing power assay systems could be used to investigate the antioxidant capacity of plant extracts. Nitric oxide is a potent pleiotropic mediator of physiological processes like neuronal signaling, inhibition of

platelet aggregation, smooth muscle relaxation, and regulation of cell mediated toxicity. Reactive nitrogen species (RNS), are a family of molecules derived from nitric oxide (·NO) and superoxide ($O2^{\cdot -}$) that have been implicated in carcinogenesis. Nitric oxide scavengers compete with oxygen leading to the reduced production of nitric oxide **(Subhashini *et al.*, 2011)**. Hence we tested whether our herbal extracts possessed Nitric oxide scavenging activity. The Diphenyl picryl hydrazyl radical scavenging activity is the oldest indirect method, widely used to evaluate the free radical scavenging effect of natural antioxidants. In alcoholic medium (ethanol) DPPH showed a strong violet colour which disappeared upon combination with antioxidants and is correlated with the presence of hydroxyl groups. This test is easy and takes less time to be performed and hence it was used in our study. (**Subhashini *et al.*, 2011**). Superoxide anions serve as precursors of singlet oxygen and hydroxyl radicals. The superoxide radical scavenging activity is one of the most important assay systems to demonstrate antioxidant activity of plant extracts. All these assays above mentioned are scientifically accepted standard procedures to assess the *in-vitro* antioxidant activities of drugs **(Subhashini *et al.*, 2011).**

The microorganisms selected for assessment of antimicrobial activity were *Streptococcus mutans* (MTCC 890) and fluconazole resistant *Candida albicans*. Several studies have demonstrated the role of microorganisms and fungi in carcinogenesis. Among the oral microflora *Streptococcus mutans* is the most important organism of the oral biofilm and recent studies have reported its role in carcinogenesis. Studies have also shown the role of *Candida albicans* in carcinogenesis. However the development of fluconazole resistant strains has become a major concern. Hence the present study was done to assess the

antimicrobial effect of the prepared plant extracts against *Streptococcus mutans* and fluconazole resistant *Candida albicans*. Fluconazole resistance was induced in *Candida albicans* strains (MTCC 227) (**Yan *et al*., 2008 with modifications**) and the antimycotic activity of the extracts were tested. The antimicrobial activity of the extracts was assessed by agar well diffusion method **(Perez *et al*., 1990)** and minimum inhibitory concentration was determined in the extracts by serial dilution method (**Wariso and Ebong, 1996**). The anticancer effects of the extracts were tested in SCC 25 ATCC CRL 1628 cell line by MTT assay **(Mosmann, 1983 and Carmichael *et al*., 1988)** assay to determine dosage, acridine orange and ethidium bromide staining by fluorescence microscopy (**Kasibhatla *et al*., 2006**) and DNA fragmentation assay by gel electrophoresis (**Kotamraju *et al*., 2000)** to assess apoptosis**.** The effect of test substances on cell cycle was analyzed by flowcytometry (**Axel, 2004)** and effect of the test substance on mitochondrial membrane potential was assessed by confocal microscopy (**Salido *et al*., 2007**). The anticancer effects of the respective active compounds trigonelline of *Trigonella foenum-graecum* L. (seeds), cinnamaldehyde, 4 hydroxy cinnamic acid, eugenol of *Cinnamomum verum* J . Presl (bark) and benzyl isothiocyanate of *Carica papaya* L. CO.2 strain (seeds) were also assessed by the above mentioned assays.

In the present study cisplatin was used as the reference standard for assessment of anticancer effects as it is the most common drug used for management of oral squamous cell carcinoma. The IC_{50} concentration of Cisplatin was found to be 20.21 μM (Figure 5.24(a), 5.24(b) and Table 5.10) in the present study. It was found to exhibit anticancer effects by induction of apoptosis as demonstrated by acridine orange and ethidium bromide stain (Figure 5.27 b and c) and confirmed by

DNA fragmentation (Figure 5.30). With regard to cell cycle, cisplatin produced S phase arrest (Figure 5.36 b and c). It was also found to induce apoptosis via the mitochondrial pathway by alteration of mitochondrial membrane potential as demonstrated by the JC-1 staining (Figure 5.42 c). The results of the present study are concurrent with the findings of **Zhang *et al*., (2006)** who showed S phase arrest induced by cisplatin on oral squamous cell carcinoma cell line and **Shen *et al*., (2007)** who have demonstrated apoptosis induction in oral squamous cell carcinoma cell line by cisplatin via p53, BID, cytochrome C and caspase-3.

In the present study hydroalcoholic extract of *Cinnamomum verum* J. Presl (bark) was used. Phytochemical analysis of *Cinnamomum verum* J. Presl (bark) revealed the presence of triterpenoids, flavonoids, glycosides, carbohydrates, polyphenols, tannins, saponins. (Table 5.2). Our results are similar to the findings of **Kala *et al.*, (2011)**.The variations in phytochemical constituents can be attributed to the solvent used and method of extraction. Quantification of cinnamaldehyde, 4 hydroxy cinnamic acid and eugenol in the aqueous, ethanol and hydroalcoholic extracts were done by HPTLC. We observed that the hydroalcoholic extracts demonstrated higher quantity of the active compounds 0.17238 % of cinnamaldehyde, 1.5667% of 4 hydroxy cinnamic acid and 0.3971% of eugenol (Figure 5.18(a), 5.18(b) and Table 5.6) based on the polarity of solvents used in combination for extract preparation. The other important phytochemicals such as saponins, polyphenols and tannins were also quantified in the extract by HPLC and UV method respectively. The results demonstrated presence of 0.31% saponins (Figure 5.44a, 5.44b and Table 5.13), 22.5 % polyphenols (Figure 5.45 and Table 5.14) and 16.88% tannins in hydroalcoholic extract of *Cinnamomum verum* J. Presl (bark) (Figure 5.46 and Table 5.15).

With regard to the antioxidant activity, *Cinnamomum verum* J. Presl extracts, we have observed a steady increase in antioxidant activity in a dose dependent manner from 10µg/ml to 1000µg/ml in all the five assay systems Total antioxidant activity assay (Figure 5.8), Reducing power assay (Figure 5.9), Nitric oxide scavenging activity assay (Figure 5.10), Diphenyl picryl hydrazyl scavenging activity assay (Figure 5.11), and Superoxide radical scavenging activity (Figure 5.12) assay were concurrent with **Peng *et al.*, (2008), Khuwijitjaru *et al*., (2012), Chia-wen *et al.*, (2009); Mathew and Abraham, (2006); Sayid and Hend, (2010)**, **Wondrak *et al*., (2010) and Yuce *et al*., (2012).** The free radical scavenging activity of the extracts could be attributed to the presence of polyphenols like catechin, epicatechin, caffeic acid and procyanidin B2 which exhibit free radical scavenging activity by hydrogen donation **(Peng *et al*., 2008).** The reducing power of the extract could be attributed to the presence of di and monohydroxyl substitutions in the aromatic ring of the flavoring compounds that could also be responsible for the hydrogen ion donation. **(Mathew and Abraham, 2006).** The presence of eugenol as the major aromatic compound in the extract could be responsible for the Diphenyl picryl hydrazyl radical scavenging activity mediated through hydrogen donation. The antioxidant property of *Cinnamomum verum* J. Presl extract could also be due to the presence of polyphenolic compounds like cinnamic acid, cinnamaldehyde, coumarin, cinnamyl alcohol, caffeic acid ferulic acid, protocatechuic acid, p coumaric acid, and vanillic acid **(Khuwijitjaru *et al*., 2012).** Cinnamaldehyde, the active compound could potentiate antioxidant properties of the extract by activating NrF2 dependent antioxidant response **(Wondrak *et al*., 2010)**.

On assessment of antimycotic effects of *Cinnamomum verum* J. Presl (bark), the extract exhibited antimycotic effects against fluconazole resistant *Candida albicans* with a zone of inhibition ranging between 16 and 18mm (Figure 5.13B, and Figure 5.14) and minimum inhibitory concentration of 15.62 µg/ml (Table 5.3). Standard fluconazole did not exhibit antimycotic effect as the strains were resistant to fluconazole. The results are concurrent with the findings of (**Prabuseenivasan *et al*., 2006; Bushra *et al*., 2005 and Ofentse *et al*., 2015).** The antimycotic activity of the extract could be attributed to the presence of cinnamaldehyde that affects sterol biosynthesis and ATPase activity in the fungal cell wall irreversibly leading to H^+ ion accumulation that alter cellular pH and finally cell death. (**Ferhout *et al*., 1999; Taguchi *et al*., 2012; Shreaz *et al*., 2011, Shreaz *et al*., 2013 and Jantan *et al*., 2008).** With regard to antimicrobial effect against *Streptococcus mutans*, the zone of inhibition was absent at 250 µg/ml (Figure 5.15B and Figure 5.16) and was evident at a higher concentrations whereas the standard kanamycin exhibited higher zone of inhibition at a lower concentration of 30µg/ml (Table 5.4). It could hence be inferred that hydroalcoholic extract of *Cinnamomum verum* J. Presl (bark) exerts higher antimycotic effects than antibacterial effects. The factor could be attributed to the seasonal, geographic variations in plant collection and solvent used for extraction.

With regard to the anticancer effects, the results of the MTT assay revealed an IC_{50} concentration of 16.36µg/ml (Figure 5.23(a), 5.23(b) and Table 5.9). Previous studies by **Chulasiri *et al*., (1984)** demonstrated an IC_{50} concentration of 60 and 58 µg/ml in KB cells and 24 and 20 µg/ml in L1210 cells when petroleum ether and chloroform extracts of *Cinnamomum verum* J. Presl. were used respectively. The variations in the IC_{50} concentrations obtained in the present study

could be attributed to the geographic and seasonal variations of plant collection, solvents used for extract preparation and property of the cell line.

Cinnamomum verum J. Presl. (bark) was found to induce apoptosis in oral squamous cell carcinoma cell line as demonstrated by acridine orange and ethidium bromide stain (Figure 5.25d and 5.25e) and confirmed by DNA fragmentation (Figure 5.29). Cell cycle analysis by flowcytometry revealed that the extract induced S phase arrest (Figure 5.32 b and c) and JC-1 staining (Figure 5.43a) revealed that the extract induced apoptosis via alteration of mitochondrial membrane potential. The results are concurrent with the findings of **Manal *et al*., (2012)** who have demonstrated anticancer effects of *Cinnamomum verum* J. Presl on hepatocellular carcinoma.

Anticancer effects of the respective active compounds cinnamaldehyde, 4 hydroxy cinnamic acid, eugenol of *Cinnamomum verum* J. Presl (bark) were assessed on the same cell line. These active compounds exhibited an IC_{50} concentration of 15.64µM, 14.51µM, 24.71 µM respectively (Figure 5.24(a), 5.24(b) and Table 5.10). The compounds exhibited anticancer effect via S phase arrest (Figure 5.38 b and c, Figure 5.39 b and c, Figure 5.40 b and c), induction of apoptosis demonstrated by acridine orange and ethidium bromide stain (Figure 5.28 a, b, c, d, e and f) and confirmed by DNA fragmentation (Figure 5.30). The results of the present study with regard to cinnamaldehyde differ with that of **Shih-Hua *et al*., (2004)** who have demonstrated G2M phase arrest induced by Trans cinnamaldehyde on Jurkat and U937 cell line and **Kim *et al*., (2010)** who have demonstrated G2M phase arrest induced by 2 hydroxy cinnamaldehyde on oral

cancer cell line SCC15 and Hep-2. This could be attributed to the difference in the chemical structure of the compound and the property of the cell line.

With regard to 4 hydroxy cinnamic acid, our findings are concurrent with the findings of **Niero and Machado-Santelli, (2013)** who have demonstrated apoptosis induction on HT144 melanoma cell line. However the study contradicts the findings of **Jian *et al*., (2014)** who have reported G0/ G1 arrest induced by transcinnamic acid on K562 cell lines. This variation could be partly explained due to the difference in chemical structure and the type of the cell line studied. The findings on the anticancer effects of eugenol in the present study are concurrent with the findings of **Kim *et al*., (2006); Ghosh *et al*., (2005)** who have reported similar effects on melanoma cell line**.** A contradictory finding by **Rothschild, (1970)** who have reported G2M arrest induced by eugenol. However their study used methoxyestradol and eugenol in combination that is different from the present study. Several other phytochemical constituents in *Cinnamomum verum* J. Presl extract like saponins, polyphenols and tannins have anticancer effects. While saponins cause apoptosis induction (**Man *et al*., 2010**), polyphenols and tannins cause cell cycle arrest, regulates carcinogen metabolism, inhibits DNA binding, proliferation, cell adhesion, migration and differentiation, blocks signaling pathways. **(Huang *et al*., 2009).**

With regard to *Carica papaya* L. (seeds) hydroalcoholic extracts of the same were used for the present study. Phytochemical analysis of *Carica papaya* L. (seeds) showed the presence of triterpenoids, flavonoids, steroids, glycosides, polyphenols, saponins, amino acids. (Table 5.2). Our results are similar to the findings of **Subal *et al.,* (2010)** Minor variations in phytochemical constituents can

be attributed to the solvent used and extraction procedure. On quantification of benzyl isothiocyanate in the aqueous, ethanol and hydroalcoholic extracts by HPTLC, the hydroalcoholic extracts demonstrated higher quantity of the active compound of 0.283 % (Figure 5.19(a), 5.19(b) and Table 5.7) based on the polarity of solvents used in combination for extract preparation.

Antioxidant activity assays such as Total antioxidant activity assay (Figure 5.8), Reducing power assay (Figure 5.9), Nitric oxide scavenging activity assay (Figure 5.10), Diphenyl picryl hydrazyl scavenging activity assay (Figure 5.11) and Superoxide radical scavenging activity (Figure 5.12) assay revealed activity in all the assays in a dose dependent manner. The results were statistically significant and concurrent with those of **Norshazilla *et al.*, (2010), Osato *et al.,* (1993) and Zhou *et al.,* (2011)** who have demonstrated the *in-vitro* antioxidant activity of *Carica papaya* L. (seed) extract through various model systems. The antioxidant property could be attributed to the presence of polyphenols and flavonoids (Table 5.2) that act as metal chelators and break the reaction of free radicals. The reducing power, free radical scavenging activity and superoxide radical scavenging activity could be attributed to the presence of active phenolic compounds such as vanillic acid and parahydroxybenzoic acid (**Zhou *et al*., 2010**) that are known to neutralize singlet and triplet oxygen molecules.

On assessment of antimycotic activity of *Carica papaya* L. (seed) extracts against fluconazole resistant *Candida albicans,* a zone of inhibition ranging between 16-24 mm (Figure 5.13E and Figure 5.14) and a minimum inhibitory concentration of 15.62 µg/ml (Table 5.3) was observed in the present study. The antimycotic property could be attributed to the presence of 2,3,4 trihydroxytoluene a

potent antifungal compound (**Onkar and Ali, 2011)**. Moreover the presence of benzyl isothiocyanate could also confer fungicidal property to the extracts as isothiocyanates form thiocarbazones and thioureas on reaction with thiol and amino groups leading to inhibition of proteins and enzymes required for survival of fungal and bacterial cell. With regard to the antimicrobial effects against *Streptococcus mutans* the zone of inhibition was absent at 250 μg/ml (Figure 5.15E, Figure 5.16) and evident at higher concentrations whereas standard kanamycin exhibited higher zone of inhibition at a lower concentration of 30 μg/ml (Table 5.4). The mild antimicrobial activity could be partly due to the solvent used for extraction (**Ogunjobi and Ogunjobi, 2011**) and the seasonal, geographical variations that affect the concentration of biologically active components.

In the present study treatment of oral squamous cell carcinoma cell line with *Carica papaya* L. (seed) extract an IC_{50} concentration of 62.49 μg/ml (Figure 5.23(a), 5.23(b) and Table 5.9) was observed. The extract exhibited anticancer effects by induction of apoptosis demonstrated by acridine orange and ethidium bromide stain (Figure 5.26e and 5.26f). The results were confirmed by DNA fragmentation (Figure 5.29). Cell cycle analysis by flowcytometry revealed G2M and S phase arrest. (Figure 5.35 b and c). The apoptosis induction was via the mitochondrial pathway as demonstrated by green fluorescence on staining with JC-1 (Figure 5.43d). Similar studies by **Li *et al*., (2012) and Nakamura *et al*., (2007)** have reported the anticancer effects of *Carica papaya* L. seed and benzyl isothiocyanate on H60 and HL060 cell line respectively. Moreover the findings of **Nakamura *et al*., (2007)** who have reported the anticancer effects of *Carica papaya* L. (seed) and benzyl isothiocyanate HL-60 cell line via apoptosis induction are concurrent with the results of the present study.

The anticancer effects of the respective active compound benzyl isothiocyanate of *Carica papaya* L. (seed) was also assessed. Benzyl isothiocyanate demonstrated an IC_{50} of 29.80µM (Figure 5.24(a), 5.24(b) and Table 5.10) in the MTT assay. The oral squamous cell carcinoma cells treated with benzyl isothiocyanate were stained with acridine orange and ethidium bromide. An increased proportion of apoptotic cells were evident in comparison with the extract (Figure 5.28 g and h). The results were confirmed by the DNA fragmentation assay (Figure 5.30). Cell cycle analysis by flowcytometry revealed significantly increased number of cells in the Sub G0 phase (Figure 5.41 b and c). Apoptosis was seen to occur via the mitochondrial pathway as indicated by green fluorescence in JC -1 stain (Figure 5.42 d). The anticancer effects of benzyl isothiocyanate on several cell lines other than oral squamous cell carcinoma have been reported by various authors **Kim *et al*., (2011), Hecht *et al*., (2002), Liu *et al*., (2011) and Sahu and Srivastava, (2009)**. On the contrary the studies done by **Antony *et al*., (2012), Wu *et al*., (2011) and Huang *et al*., (2012)** have reported that benzyl isothiocyanate induced G2M arrest on MDA-MB-231 (breast), MCF-7 (breast), and HCT-116 (colon) human cancer cells, Human osteogenic sarcoma U-2 OS cells and A375.S2 human melanoma cancer cells. Thus these variations could partly be attributed to the cell line studied. On comparison of the effect of the extract and active compound we have observed that the extract induced G2M and S phase arrest whereas the active compound demonstrated increased proportion of cells in the Sub G0 population. The variation in results obtained with the extracts and pure compounds could be due to the cumulative effect of other phytochemicals present in the extract.

In the present study, hydroalcoholic extract of *Carica papaya* L. (leaves of male and female plant) was used to assess the antioxidant and antimicrobial

activity. Phytochemical analysis of leaves of male plant of *Carica papaya* L. showed presence of flavonoids, glycosides, carbohydrates, polyphenols, and saponins (Table 5.2). Interestingly, leaves of female plant of *Carica papaya* L. showed presence of all the phytochemical constituents of male leaves except saponins (Table 5.2). To our knowledge this is the first study to have assessed the difference in phytochemical constituents of papaya leaves of male and female plant.

On assessment of antioxidant activity *Carica papaya* L. (leaves of male and female plant) exhibited significant antioxidant activity in Reducing power assay (Figure 5.9), Nitric oxide scavenging activity assay (Figure 5.10), Diphenyl picryl hydrazyl scavenging activity assay (Figure 5.11) and Superoxide radical scavenging activity (Figure 5.12) assay with a steady increase from 10µg/ml to 1000µg/ml. With regard to Total antioxidant activity assay, *Carica papaya* L. (leaves of male plant) showed an increase in the activity from 10µg/ml to 1000µg/ml while leaves of female plant showed steady increase in activity from 50µg/ml to 1000µg/ml and slight increase at 10µg/ml (Figure 5.8). Our results were statistically significant. The reducing power, radical scavenging activity and superoxide anion scavenging activity exhibited by *Carica papaya* L. (leaves) could be attributed to the presence of polyphenols and flavonoids (Table 5.2) which are reducing agents and singlet oxygen scavengers. Compounds like papain, chymopapain, cystatin, tocophenol, ascorbic acid and cyanogenic glucosides of *Carica papaya* L. can confer antioxidant property as they can increase the total antioxidant power in blood and significantly reduce lipid peroxidation level **(Otsuki *et al.*, 2010**). Our study results are concurrent with the findings of **Srikanth *et al.* (2010); Indran *et al.*, (2008) and Iyawe, (2011)** who have demonstrated a significant antioxidant activity of *Carica papaya* L. (leaf) extract. However the present study is novel because we have

separately analyzed male and female plant extracts. So far no study has analyzed the differences between male and female leaf properties. This difference could be an outcome of sexual dimorphism in plants that could cause a difference in phytochemical constituents.

In the present study, when the antimycotic activity of *Carica papaya* L. (leaves of male and female plant) against fluconazole resistant *Candida albicans* were assessed, we have observed a zone of inhibition ranging between 11 and 15 mm (Figure 5.13C, 5.13D and 5.14) and minimum inhibitory concentration of 15.62 µg/ml (Table 5.3). The results of the present study are concurrent with the findings of **Nwachukwu and Umechuruba, (2001) and Chávez-Quintal *et al*., (2011).** The terpenes, and lipophilic flavonoids of *Carica papaya* leaves could confer fungicidal effects by interaction with fungal cell membrane, the flavonoids could exert antimicrobial effects by inhibiting enzyme activity by forming complex with bacterial cell wall, extra cellular and soluble proteins, saponins exert antimicrobial effects via facilitating the entry of toxic material into the cells and leakage of vital nutrients from the cells of microorganisms. With regard to the antimicrobial effects against *Streptococcus mutans* the zone of inhibition was absent at 250 µg/ml (Figure 5.15C, 5.15D, and 5.16) and evident at higher concentrations whereas standard kanamycin exhibited higher zone of inhibition at a lower concentration of 30 µg/ml (Table 5.4). Thus it can be inferred that hydroalcoholic extracts of *Carica papaya* L. (leaves of male and female plant) exhibit higher antimycotic than antimicrobial activity. This could be partly attributed to the solvent used for extraction as reported by **Okunola *et al*., (2012)** that organic solvents had better antimicrobial activity than aqueous extract.

In the present study, the alkaloid fraction of *Carica papaya* L. (leaves of male and female plant) was isolated and quantified. The results indicated that leaves of male plant contained 7.69% and female plant contained 7.39% of alkaloids (Table 5.8). The Nitric oxide radical scavenging activity of the alkaloid fraction was assessed and compared with the hydroalcoholic extracts. We have observed that the alkaloid fraction demonstrated higher antioxidant activity than the hydroalcoholic extract (Figure 5.21). Moreover a study by **Do Thi. H and Do Thi. T, (2013)** showed that the alkaloid fraction of *Carica papaya* L. (leaf) exhibited greater anticancer effects than the flavonoids, saponins and other polar compounds on breast cancer cells MCF7, carcinoma cell line KB, lung cancer cell line LU-1and leukemia cell line HL-60.Hence, we have used the alkaloid fraction of *Carica papaya* L. (leaves of male and female plant) to assess the anticancer effects.

The alkaloid fraction of *Carica papaya* L. (leaves of male and female plant) demonstrated an IC_{50} concentration of 85.16 and 90 µg/ml respectively (Figure 5.23(a), 5.23(b) and Table 5.9). Both the extracts exhibited anticancer effects via induction of apoptosis as demonstrated by acridine orange and ethidium bromide stain (Figure 5.26 a, b, c and d) and confirmed by DNA fragmentation (Figure 5.29). With regard to cell cycle analysis *Carica papaya* L. (leaves of female plant) exhibited G2M and S phase arrest (Figure 5.34 b and c) and *Carica papaya* L. (leaves of male plant) exhibited S phase arrest (Figure 5.33 b and c). This interesting difference was also a first time finding. Both the extracts were found to induce apoptosis via mitochondrial pathway as evident by the JC- 1 staining results (Figure 5.43 b and 5.43 c). Previous studies by **Morimoto *et al*., (2008); Rumiyati, (2006) and Otsuki *et al.*, (2010)** have shown the anticancer effect of papaya leaf extract on

several cell lines other than oral squamous cell carcinoma. Our study results are concurrent with the findings of **Karthik *et al*., (2012)** who have reported that the alkaloids exert anticancer effects via apoptosis induction. The alkaloids that have been isolated from *Carica papaya* L. (leaves) that could confer anticancer effects are carpaine, psuedocarpaine and nicotine.

In the present study, hydroalcoholic extracts of *Trigonella foenum-graecum* L. (seed) were used. *Trigonella foenum- graecum* L. (seeds) showed the presence of triterpenoids, flavonoids, steroids, glycosides, carbohydrates, alkaloids, polyphenols, saponins and amino acids (Table 5.2). Our results are consistent with the findings of **Priya *et al*., (2012).** On quantification of trigonelline hydrochloride in the aqueous, ethanol and hydroalcoholic extracts of *Trigonella foenum-graecum* L. by HPTLC, the hydroalcoholic extracts demonstrated higher quantity of the active compound of 2.21 % (Figure 5.17a, 5.17b and Table 5.5) based on the polarity of solvents used in combination for extract preparation.

Antioxidant activity assays such as Total antioxidant activity assay (Figure 5.8), Reducing power assay (Figure 5.9), Nitric oxide scavenging activity assay (Figure 5.10), Diphenyl picryl hydrazyl scavenging activity assay (Figure 5.11) and Superoxide radical scavenging activity (Figure 5.12) assay revealed a significant activity in all the assays in a dose dependent manner with a steady increase in activity from 10µg/ml to 1000µg/ml. The antioxidant properties of *Trigoenella foenum-graecum* L. (seeds) could be attributed to the presence of polyphenols, flavonoids and saponins (Table 5.2). Phenolic compounds such as naringenin, quercetin **(Kaviarasan *et al.,* 2007)** and coumarin **(Syeda *et al*., 2008)** have been shown in *Trigoenella foenum-graecum* L. (seeds) that possess good

hydrogen donating ability. Flavonoids like vixetin, quercetin, naringenin, tricin, and tricin 70 beta glucopuranoside (**Shang *et al.*, 1998)** present in *Trigonella foenum-graceum* L. (seeds) possess hydroxyl groups that can scavenge free radicals by hydrogen ion donation and are good metal chelators. Presence of saponins could also cause a decrease in lipid peroxidation. Our study results are concurrent with the findings of **Subhashini *et al*., (2011); Priya *et al*., (2011); Kaviarasan *et al*., (2007); Syeda *et al*., (2008); Acharya *et al*., (2011); Ravikumar and Anuradha, (1999) and Thirunavukkarasu *et al*., (2003)** who have reported the antioxidant property of *Trigonella foenum-graecum* L. (seeds).

With regard to antimycotic activity, it was found that *Trigonella foenum-graecum* L. (seeds) exhibited antimycotic activity against fluconazole resistant *Candida albicans* with a zone of inhibition ranging between 10 and 14 mm (Figure 5.13A and 5.14) and minimum inhibitory concentration of 15.62 µg/ml (Table 5.3). The antimycotic activity of the seeds could be attributed to the presence of bioactive oils, volatile oils and alkaloids. A study by **Omezzine *et al*., (2014)** has shown the antimycotic activity of *Trigonella foenum-graecum* L. during various stages of development and level of ploidy and have shown that five novel compounds 3-O-b-D-glucopyran- oside, kaempferol 7-O-glucoside, kaempferol 3-O-a-L-rhamnosyl (1fi2) b-D-xyloside, kaempferol 7-O-b-D-glucopyranosyl (1–4) b-D-glucopyranoside and kaempferol 3-O-b-glucosyl (1fi2) could be responsible for antimycotic property of the herb. With regard to *Streptococcus mutans* the zone of inhibition was present only at 1000 µg/ml (Figure 5.15A, Figure 5.16) and absent at lower concentrations whereas the standard kanamycin exhibited higher zone of inhibition at a lower concentration of 30µg/ml (Table 5.4). The mild antimicrobial activity could be attributed to the solvent used for extraction. Studies by **Walli *et al*.,**

(2015); Mawahib *et al*., (2015) and Abdel-Massih *et al*., (2010) have reported that boiling water extract, petroleum ether extract and aqueous extract of *Trigonella foenum- graecum* L. (seeds) respectively exhibited higher antimicrobial and antimycotic properties.

In the present study, hydroalcoholic extracts of *Trigonella foenum-graecum* L. (seeds) demonstrated an IC_{50} concentration of 39.70µg/ml (Figure 5.23(a), 5.23(b), Table 5.9). The extract exhibited anticancer by effects by induction of apoptosis demonstrated by acridine orange and ethidium bromide stain (Figure 5.25 b and c), however it was not evident in DNA fragmentation assay (Figure 5.29). With regard to its effects on cell cycle it exhibited S phase arrest (Figure 5.31 b and 28c). The results of the present study are concurrent with the findings of **Shaban *et al*., (2009)** who have demonstrated anticancer effects of *Trigonella foenum-graecum* L. (seeds) via apoptosis induction. **Shabbeer *et al*., (2009)** have reported that the ethanolic extracts induced cell death in LNCaP cell line, G2M arrest in PC 3 cells, down regulation of p53 in DU145 cells, down-regulation of TGF beta and p21 induction in Pc 3 cells activation of IGF 1 receptor in DU145 cells. This implicates different mechanism of action of the same substance in different cell lines.

The respective active compound trigonelline hydrochloride demonstrated an IC_{50} concentration of 20.07 µM, (Figure 5.24(a), 5.24(b) and Table 5.10) and exhibited anticancer effects via by induction of apoptosis demonstrated by acridine orange and ethidium bromide staining (Figure 5.27 d and e) confirmed by DNA fragmentation assay (Figure 5.30) and S phase arrest (Figure 5.37 b and c) **Arlt *et al.*, (2012)** have reported that trigonelline sensitizes pancreatic cancer cells for apoptosis. Thus the anticancer effects of *Trigoenlla foenum-graecum* L. (seeds)

could be attributed to the presence of trigonelline. The absence of ladder pattern in DNA fragmentation representing mild apoptotic activity of the extract could be attributed to the concentration of trigonelline in the extract in comparison with the pure compound as the concentration of the biologically active agents could vary with geographical region of harvest, condition of soil, harvest season **(Shabber *et al*., 2009).**

The apoptotic activity of trigonelline and *Trigonella foenum-graecum* L. (seeds) was least in comparison with the other extracts. Hence, the anticancer activity could be via different mechanism like suppression of ROS potentiated invasive effects on hepatoma cell line (**Hirakawa *et al*., 2005)**.

As described previously we have used five different assays to evaluate the antioxidant properties of herbal extracts. With regard to Total antioxidant activity assay (Figure 5.8), and Superoxide radical scavenging activity assay (Figure 5.12), *Carica papaya* L. (leaf of female plant) exhibited maximum antioxidant activity at a concentration of 1000µg/ml in comparison with other extracts. Similarly *Carica papaya* L. (seeds) showed highest Diphenyl picryl hydrazyl radical scavenging activity (Figure 5.11) at 1000µg/ml. Reducing power (Figure 5.9) and Nitric oxide scavenging activity assay (Figure 5.10) showed maximum efficacy for *Trigonella foenum-graecum* L. (seed) extracts at 1000µg/ml. To the best of our knowledge this is the first study to have compared the antioxidant activities of *Trigoenlla foenum-graecum* L. (seeds), *Cinnamomum verum* J. Presl (bark) and *Carica papaya* L. (leaves of male plant, female plant and seed) extracts. The above results shed light on the fact that each herbal extract exerts its antioxidant effect

through different mechanisms. Hence, a combination of these would possibly have a synergestic effect in mitigating oxidative stress.

With regard to the antimicrobial activity against fluconazole resistant *Candida albicans*, *Carica papaya* L. (seed) extract exhibited highest zone of inhibition at all concentrations in comparison with the other extracts. *Cinnamomum verum* J. Presl (bark) extract exhibited highest zone of inhibition at 1000µg/ml against *Streptococcus mutans*. To the best of our knowledge this is the first study to have compared the antimicrobial activities of *Trigonella foenum-graecum* L. (seeds), *Cinnamomum verum* J. Presl (bark), *Carica papaya* L. (leaves of male and female plant and seed) extracts.

With regard to the anticancer activity *Cinnamomum verum* J. Presl (bark) demonstrated anticancer effects and was effective at a low IC_{50} concentration in comparison with the other extracts. This could be attributed to the combined effects of the important phytochemical constituents such as cinnamaldehyde, 4 hydroxy cinnamic acid, eugenol, polyphenols and tannins present in the extract.

It is a well-known fact that the herbs used in the present study have been a part of the Indian diet since several hundred years. All the herbs and the respective active compounds demonstrated anticancer activity were potent at different concentrations. In addition to this, the antimicrobial and antimycotic property of the extracts can alter the oral microflora thereby reduce the risk for cancer and alter the biological behavior of cancer cells. The antioxidant activity of the herbal extracts could also play a dual role in prevention of carcinogenesis as well as cancer therapy.

A polyherbal formulation containing all the extracts could be done to assess synergestic effect.

Hence, the combined effect of the decreasing free radical formation (antioxidant), suppression of microbial growth (antimicrobial and antimycotic), induction apoptosis and suppression of proliferation in cancer cells should be further explored for management of oral squamous cell carcinoma and prevention of recurrence. These effects could also be explored for biochemoprevention. However the limitations of the study is that it has an *in-vitro* design and studies to evaluate toxicity and *in-vivo* anticancer effects have to be carried out.