5. Discussion

Medicinal and aromatic plants are the largest source of bioactive compounds on Earth that have been used by humans for treating various diseases since ancient times. Plant based products have also played an important role in cancer treatment. Vincristine, vinblastine, taxol are some examples of plant based drugs being used for cancer therapy. The natural products are easily available, cost effective, and less toxic as compared to the allopathic drugs available for cancer treatment. As a result, extensive screens for plant based medicines were initiated worldwide (Solowey et al., 2014). Such anticancer screens have largely been performed in cultured cell systems and animal model systems (HogenEsch and Nikitin, 2012). The cell lines do not represent the vast heterogeneity of the tumor and the heterogeneous nature of tumours from one patient to another. Therefore, multiple cell lines would be required to study the full heterogeneity in a tumour phenotype, which is highly cost intensive. Another biological system amenable for screening anticancer compounds is the budding yeast, *Saccharomyces cerevisiae* has been successfully used as a screening tool for the identification of bioactive molecules using high-throughput halo assay (Gassner et al., 2007). As many cellular processes are highly conserved between yeast to human, the bioactive compounds showing inhibitory effects in yeast can be used for discovery of similar processes in human subjects. Based on this concept, the present study was undertaken to isolate and characterize phytocompounds from medicinal and aromatic plants of Himachal Pradesh for their anti-proliferative effects on budding yeast cell cycle. Owing to the conservation of cell cycle machinery between yeast and humans, which is perturbed in all cancers, phytocompounds identified in yeast screen will be a direct candidate for anticancer therapy in humans. A comparison of the results from the present study with the reports existing in literature are discussed below:

5.1 *S. cerevisiae* as a model system for screening anti proliferative activities

*S. cerevisiae* has been used as a system for drug discovery (Barberis et al., 2005), but rarely for anti-proliferative compounds. There are only few reports on the use of budding yeast for anti-proliferative activities (Saboo et al., 2012). Qaddouri et al (2011) reported that yeast cells treated with Lyc, a natural alkaloid produced fragmented nuclei,
implicating its effects on cell-cycle machinery. In the present study, a total of 62 plants (14 non medicinal, 46 medicinal and 2 aromatic plants) were screened for anti-proliferative activity against the budding yeast, *S. cerevisiae*. It was observed that extracts from 17 plants and essential oils from two aromatic plants showed antiproliferative effects. Thus, our results validate the use of *S. cerevisiae* as a tool for screening anti-proliferative activities from medicinal and aromatic plants. Our study represents the first report on screening of medicinal and aromatic plants of H.P., India for anti-proliferative activities against *S. cerevisiae*.

5.2 **Antiproliferative activity of medicinal and aromatic plants against *S. cerevisiae***

In this study, 19 out of 62 plants exhibited potent anti-proliferative activities against *S. cerevisiae*. Interestingly, of the 43 plants that failed to inhibit yeast growth, several of them have been reported in literature for anti-cancer activities, including *P. hexandrum*, *B. utilis*, *C. sinensis*, *Mentha spp.*, *W. somnifera* and *Z. officinale* (Sakarkar and Deshmukh, 2011). The failure to detect their anti-proliferative activities could be due to multiple reasons like, the difference in phytochemical composition due to geographic differences, different mechanism of anti-cancer activity, or the differences in the solvents used for plant extraction in this study. Nevertheless, more than 19 plants showed antiproliferative effect against *S. cerevisiae*.

*C. indicum* (leaves), *C. longa* (rhizome), *A. marmelos* (leaves, fruit) and *C. citratus* (oil) showed strongest antiproliferative effect against yeast growth. There are no reports of any of these plants for anti-proliferative effect on budding yeast, *S. cerevisiae*. However, their antimicrobial effects have been well studied in the related pathogenic yeast, *C. albicans* (Sharanappa and Vidyasagar, 2013) and antiproliferative effects in animal cell lines (Talib and Mahasneh, 2010). Hence, the results of the present study are discussed below in comparison to these studies:

In our study, ethanolic and acetone extracts of leaves of *A. marmelos* exhibited class 1 antiproliferative effect, with cidal effect on yeast growth, indicating that its phytocompounds kill the yeast cells. *Candida albicans* was found to be the most susceptible fungus to methanolic fruit extract of *A. marmelos* among the tested fungal
strains (Parihar and Kumar, 2013). The petroleum ether extract from leaves of A. marmelos exhibited the highest antifungal efficacy against C. albicans and other tested fungal species (Kothari et al., 2011). A molecule, 1-hydroxy-5, 7-dimethoxy-2-naphthalene-carboxaldehyde (HDNC, marmelin) was isolated from ethyl acetate fraction of extracts of A. marmelos and found to inhibit proliferation of HCT-116 (colon cancer cell line) and HEP-2 (alveolar epithelial carcinoma cell line) cells by increasing levels of activated caspase-3 and inducing arrest of treated cells at G1 phase of cell cycle (Subramaniam et al., 2008). In our study, it remains to be studied if the active phytocompound of A. marmelos causes cell cycle arrest.

In our study, ethanolic and acetone extracts of C. indicum leaves, but not its essential oil, exhibited class 1 antiproliferative effect with fungistatic effect on yeast growth. Pradhan et al. (2011) reported that essential oils from C. indicum showed maximum antifungal activity at 1% v/v against Candida albicans (zone of inhibition of 18.0 mm). In another study, C. indicum ethanolic extract inhibited the proliferation of human hepatocellular carcinoma (HCC) cells in S phase of cell cycle and induced apoptosis by increasing p21 levels and decreasing CDK4 protein expression (Li et al., 2009).

In the present study, ethanol, acetone and chloroform extracts of C. longa exhibited class 1 antiproliferative effect with strong cidal effect on yeast growth. Curcuminoids from ethyl acetate extract of C. longa rhizomes were found to be antifungal against Candida albicans, C. kruseii and C. parapsilosis (Roth et al., 1998; Harit et al., 2013). Curcumin, a compound isolated from C. longa, caused induction of apoptotic cell death and arrested human bladder cancer cells in G2/M phase (Karunagaran et al., 2005). However, in the present study, purified curcumin failed to have significant anti-proliferative effect on yeast cells (Fig. 4.3 A). C. longa is documented for anti-inflammatory (Jurenka, 2009), anticancer, antioxidant and hepatoprotective effects (Labban, 2014).

In our study, essential oil of C. citratus leaves exhibited class 1 antiproliferative activity, with a strong cidal effect on yeast growth and arrested growth of cells in G2/M.
phase of cell cycle. Citral, 3,7-dimethyl-2,6-octadienal, a key component of the essential oil of *C. citratus* was observed to induce apoptosis in acute promyelocytic leukemia cell line (NB4) (Hailong *et al*., 2013) and inhibited caspase-3 in tumors cell lines (Dudai *et al*., 2005). Citral treatment caused inhibition of MCF-7 breast cancer cells by arresting treated cells in G2/M phase (Chaouki *et al*., 2009).

In the present study, essential oil of *R. officinalis* leaves showed moderate anti-proliferative effects with cidal effect on yeast growth. The budding index count data revealed that *R. officinalis* oil causes arrest of yeast cells at G2/M transition of cell cycle. Antimicrobial activity of the essential oil, and methanolic extract of *R. officinalis* against *S. cerevisiae* and *Candida krusei* was reported by Tavassoli (2011). The antiproliferative effect of the polyphenols, carnesol and carnosic acid present in *R. officinalis* was reported against colorectal cancer cells (Visanji *et al*., 2006). The phytocompounds were found to arrest treated cells in the G2/M phase of the cell cycle. Carnesol caused an increase in cyclin B1 protein levels, whereas carnosic acid caused reduction in cyclin A levels.

### 5.3 Phytochemical screening of extracts and essential oil of plants with antiproliferative effect on yeast cells.

The class 1 plants listed in Table 4.2 that exhibited antiproliferative activity were analyzed for the presence of valuable phytochemical constituents in the plant extracts and essential oil. *C. longa* ethanolic extract showed presence of terpenoids, steroids, flavanoids, tannins, sugar and phenols. Swadhini *et al*. (2011) reported the presence of flavanoid, glycosides, phenolics and alkaloid, and absence of tannin and saponins. Chhetri *et al*. (2008) reported the presence of terpenoids, flavanoids and glycosides, and absence of alkaloids, tannin and glycosides.

In this study, *A. marmelos* ethanolic extract of leaves or fruit was observed to contain glycosides, terpenoids, steroids, flavanoids, tannins, sugar and phenols. In other studies, alkaloid and saponins were present, and sugar was absent (Venkatesan *et al*., 2009; Chanda and Pattabhiramaiah, 2014).

In this study, *C. indicum* ethanolic extract of leaves showed the presence of glycosides, terpenoids, steroids, flavanoids, tannins, sugar and phenols. The crude
methanolic extracts of *C. indicum* leaves showed the presence of alkaloid, flavanoids and terpenoids (Yadav *et al.*, 2010), but alkaloids were not detected in our study.

The terpenoids in *R. officinalis* essential oil were responsible for antiproliferative effect against budding yeast in our study. We detected six phytocomponents from *R. officinalis* oil in this study; viz. glycosides, terpenoids, steroid, flavanoids, sugar and phenol. Asressu (2013) reported the presence of flavanoids, terpenoids and phenolics in methanolic *R. officinalis* extracts.

Phytochemical analysis of *C. citratus* oil indicated the presence of alkaloids, glycosides, terpenoids, steroids, flavanoids, tannins, sugar, saponins and coumarins in this study. *C. citratus* essential oil was shown positive for the presence of terpenes, alcohols, ketones, aldehyde and esters (Shah *et al.*, 2011).

**5.4 Effect of *R. officinalis* and *C. citratus* essential oil on the budding index of yeast cells**

Essential oils have been used for their antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Bassole and Juliani, 2012). However, essential oils have not been explored for their antiproliferative effects in budding yeast. In this study, essential oil of two aromatic plants, *C. citratus* and *R. officinalis* exhibited antiproliferative effect on yeast cells by mediating cell cycle arrest at G2/M transition. Although, cell cycle analysis data exist for a lot of phytocompounds (as described in 6.2), there are very few reports on such studies in yeast like *S. cerevisiae*. Thus, our study forms the first report on the cell cycle arrest of *S. cerevisiae* at G2/M transition caused by essential oils of *C. citratus* and *R. officinalis*.

**5.5 Characterization of essential oil of *R. officinalis* and *C. citratus***

*R. officinalis* oil used in our study was characterized by GC/MS, which revealed that carene (7.75%), pinene (15.1%), camphene (6.15%), Bicyclo[3.1.1]hept3en2one,4,6,6trimethyl, (1S)- (7.16%), and 2Cyclohexen1ol, 1methyl4(1methyl ethyl),Trans (8.94 %) are the predominant constituents. In another study on *R. officinalis* oil, p-cymene (44.02%), linalool (20.5%), gamma-terpinene (16.62%), thymol (1.81%), beta-pinene (3.61%), alpha-pinene (2.83%) and eucalyptol
(2.64%) were identified as major constituents (Ozcan and Chalchat, 2008). Tavassoli et al. (2011) reported the major constituents of *R. officinalis* oil to be 1,8-Cineole (23.14%), camphor (12.35%), -pinene (9.87%), -pinene (6.10%), borneol (5.61%), camphene (5.58%) and -terpineol (4.30%). Such differences could be due to the variations in the location and time of collection.

The essential oil of *C. citratus* or lemon grass oil, consists of high percentage of citral (73.3%), neral (31.3%), and geranial (42.0%), followed by α-phellandrene (6.9%), geraniol (2%), neryl acetate (1.7%) and linalool (1.2%) (Mahanta et al., 2007).

### 5.6 Antiproliferative effect of α-pinene and carene, the major constituents of *R. officinalis* essential oil

In this study, the active phytocompounds of essential oil from *R. officinalis* responsible for anti-proliferative effect on yeast cells were found to be the terpenoids, α-pinene and carene. Both α-pinene and carene showed enhanced anti-proliferative effect as compared to the essential oil of *R. officinalis* (Fig. 4.2 C and 4.9 A, B) in well diffusion assays. In addition, α-pinene and carene also mediated G2/M arrest of yeast cell cycle. These results affirm the fact that the anti-proliferative effect of *R. officinalis* essential oil is due to its constituents, carene and α-pinene.

Terpenoids constitute the largest class of natural products which are promising for cancer therapy (Huang et al., 2012). Pinene and carene have been well studied for their anti-microbial properties (Rivas da Silva et al., 2012). However, there are no reports on their anti-proliferative activities on *S. cerevisiae*.

In this study, these compounds arrested the yeast cells in G2/M transition of cell cycle (Fig. 5.1). Thus, our study is the first report on the role of carene and α-pinene in arresting yeast cell cycle.
Fig. 5.1. A proposed mechanism of action of the phytocompounds, carene and α-pinene of *R. officinalis* essential oil in yeast cell cycle. Carene and α-pinene arrest cell cycle at G2/M transition, thereby resulting in accumulation of dumb-bell shaped yeast cells, arrested at G2/M phase.