Anti-cancer inhibition studies of naturally occurring Terpenes using in-vitro technique

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Abstract. Solvent extractions of shade dried seeds of Cyamopsis tetragonoloba, Pithecellobium dulce and Coriandrum sativum plants were executed using organic solvents. The obtained extracted crudes were subjected to the chemical analysis followed by instrumental analysis using standard procedures to identify the naturally occurring phytochemicals [1, 2, 3]. Limonene, Oleanolic acid and Linalool were obtained from ethanol extracts of Cyamopsis tetragonoloba, methanol extract of Pithecellobium dulce and acetone extract of Coriandrum sativum respectively. The compound identification were confirmed by ¹H NMR, ¹³C NMR, IR and Mass spectroscopy [4]. The anti-cancer activity of the extracted terpenes determined using in-vitro analysis on HeLa-Human cervix cell lines. The obtained IC₅₀ value of Oleanolic acid shows better biological activity against HeLa cell lines. It was also found that there is no cell lysis in HeLa cell lines in case of Limonene with concentration less than 30 μg. However, biological activity of Linalool is better in case of HeLa cell line. This suggests that Oleanolic acid and Linalool is good anti-cancer agent as reported from in vitro studies against Hela-Human cervix cell line.

Keywords. Phytochemical compounds, terpenes, in-vitro, cell lysis.

1. Introduction

Natural products have been used as a traditional medicine in almost all over world from ancient times for various human diseases. Among all known natural products having drug-like properties, Terpene (also called as terpenoids) based compounds are found as the largest class of natural products in plants. A few among them are used as medicine for treatment of various diseases including cancer, as they show high biological activities. Terpenoids are volatile substances which give fragrance to the plants and flowers. Terpenes are primarily found to occur in plants. For medical purposes and biological activities many terpenes have been used. For example: paclitaxal (Taxol) as anticancer drug. Terpene based compounds are also used as therapeutics in other diseases including cancer chemo-preventive effects, anti-microbial, anti-fungal, anti-viral, anti-hyperglycemic, anti-inflammatory, and anti-parasitic activities [5, 6]. It is evident that nature is an excellent source of effective treatments. A wide variety of plant extracts have significant anti-tumor activity. Among the compounds under investigation are the cyclic tri-terpenes, mono-terpenes, di-terpenes, and derivatives of terpenes. In addition to cytotoxic activity, many plants are effective in chemoprevention [7, 8].

The Present study is to identify and isolate terpenes from seeds of selected plants Cyamopsis tetragonoloba, Pithecellobium dulce and Coriandrum sativum plants of Vidarbha region. Isolated
compounds were then studied for anticancer activities using in-vitro technique against Hela-cell line (Human Cervix). This experiment will provide biological activities in the form of IC₅₀ values.

2. Materials and Method

2.1. Selection and drying of plant material

The plant material especially seeds of various plant were collected from different region of central part of India. Central part of India is called as CP & Berar region. This region is having huge biodiversity lots of forest areas. The seeds of plants dried with care in shade for at least one month at room temperature [9].

| Sample No. | Plant Name          | Botanical Name         | Plant Part | Drying Temperature Range |
|------------|---------------------|------------------------|------------|--------------------------|
| Sample 1   | Madras thorn/ Manilla tamarind | Pithcellobium Dulce   | Seeds      | 20 – 37°C                |
| Sample 2   | Guar/Cluster beans  | Cyamopsis Tetragonoloba | Seeds      | 20 – 37°C                |
| Sample 3   | Dhania              | Coriandrum Sativum     | Seeds      | 20 – 37°C                |

The dried seeds are finely powdered using grinder and the fine powder is stored in dark and dry container.

2.2. Preparation of crude extract

The selected plant materials (seeds) were extracted by Soxhlet apparatus using different organic solvents. The selection and use of the solvents started from Polar Solvents to Non-Polar Solvents such as methanol, ethanol, acetone, ethyl acetate and n-hexane [10].

All crude extracts obtained after extraction were then analyzed for investigating phytochemicals by using High Resolution Liquid Chromatography Mass Spectrometer (HR-LCMS) and Gas Chromatography Mass Spectrometer (GC-MS).

| S. No. | Plant Name          | Crude Extract | Technique Used | Probable Terpenes |
|--------|---------------------|---------------|----------------|-------------------|
| 1.     | Pithcellobium Dulce | Methanol      | HR-LCMS        | Oleanolic acid    |
| 2.     | Cyamopsis Tetragonoloba | Ethanol | HR-LCMS        | Limonene          |
| 3.     | Coriandrum Sativum  | Acetone       | GC-MS          | Linalool          |

3. Experimental

Chemical test were carried out on the different extracts of selected plants using standard procedure as described by Mojabetal [11], Harborne [12], Sofowora [13], Trease and Evans [14], Siddiqui and Ali [15]. All the chemical analysis were performed to detect the presence of active constituents like phenol, flavanoid, quinone, cardiac glycosides, tannin, steroid, saponin and terpenoid. These procedures are already reported by number of workers and used without any modification. The name of phytochemicals and their investigation methods are as follows:
a) Cardiac glycosides
5 ml of extract was treated with 2 ml of glacial acetic acid containing a drop of FeCl₃ solution. This was then underplayed with 1 ml conc. H₂SO₄. A brown ring of the interface indicates a deoxy-sugar characteristic of Cardenolides.

b) Flavonoids
1 ml of extract a few drops of dilute NaOH was added an intense yellow colour was produced in the plant extract which becomes colourless on addition of few drops of dilute acid indicates the presence of flavonoids.

c) Terpenoid
5 ml of each extract was mixed with 2 ml of chloroform; 3 ml of concentrated H₂SO₄ was then added to form a layer. A reddish brown precipitate colouration at the interface formed indicated the presence of terpenoids.

d) Tannin
1 ml of 5% ferric chloride to solvent free extract is added. The presence of tannin is indicated by the formation of bluish black or greenish black precipitate.

e) Saponin
The extract was diluted with 20 ml distilled water and was agitated in a graduated cylinder for 15 min. the formation of 1 cm layer of foam indicates the presence of saponin.

f) Phenol
1 ml of extract 2 ml of distilled water was added followed by few drops of 10% FeCl₃ appearance of blue or green colour indicates presence of phenols.

g) Quinone
1 ml of extract and 1 ml of concentrated H₂SO₄ was added. Formation of red colour shows the presence of quinones.

h) Steroid
1 ml of extract dissolved in 10 ml chloroform and equal volume of concentrated H₂SO₄ added by sides of test tube. The upper layer turns red and sulphuric acid layer shown yellow with green fluorescence. This indicated the presence of steroids.

Phytochemical analysis performed on different extracts of three plants shows presence of terpenes and thus the extracts were then analyzed for thin layer chromatography by using different solvent mixtures and further used for column chromatography.

3.1. Column Chromatography for isolation of terpenes
Plant 1 - The methanol extract of seeds is dissolved in n-hexane and the mixture is mixed with silica gel (60-120 mesh size) in a mortar and left for some time to get dry. The hexane soluble extract was subjected to silica gel column chromatography. It is then eluted with a mixture of CHCl₃-MeOH (1:0-0:1) which gives nine fractions. The obtained fraction number 3 was separated on silica gel column and eluted with hexane: isopropyl alcohol (98:2 – 50:50) to get a fraction of compound 1 as shown figure 2. The obtained fraction was analyzed on the basis of TLC method using Chloroform: methanol-95:5 as a developing solvent, and the spots were visualized by using iodine chamber [16-18].
Figure 1. Column Chromatography for obtaining oleanolic acid

Plant 2 – The ethanol extract (3.5 ml) was dissolved in ethyl acetate and a dry mixture is made by mixing it in silica gel. The extract mixture was packed in column with silica gel uniformly and eluted with pure n-hexane. Separation of components takes place by increasing polarity of the eluent. The separation begins with pure solvent n-hexane and ends with 10% ethyl acetate: hexane mixture.

After separation total eight fractions were collected into 10-mm× 100-mm test tubes from the column. When elution processes are completed, all of the collected fractions is examined by TLC to determine which fraction contains limonene [19-20].

Plant 3 - The acetone extract was dissolved in ethyl acetate. The mixture was added in silica gel G and kept for drying. The Column was packed with silica gel first and then with extract. The column was eluted with

i) n-Hexane (100%)

ii) n-Hexane: Ethyl acetate(98:2, 95:5, 90:10, 80:20, 70:30, 50:50, 30:70, 20:80,10:90, 5:95, 2:98)

Thereafter by evaporation process, solvent was removed from all the collected fractions at room temperature. After evaporation of solvent from the fractions F3 and F4, colorless liquid were separated which is analyzed as linalool (Compound 3) by TLC followed by IR spectroscopic analysis [21].

3.2. Instrumental Techniques Performed

Compound 1, 2 and 3 obtained by column chromatography technique was identified and confirmed by different spectroscopy methods i.e. IR (Infrared spectroscopy), NMR (Nuclear magnetic spectroscopy) and Mass spectroscopy.
Figure 2. IR spectra of Oleanolic Acid

Figure 3. IR spectra of Limonene
From all above experimental process and calculations, three terpene based compounds are confirmed and verified. They are reported in table 3.

Table 3. Verified terpene based compounds.

| Sample No. | Compound Name | IUPAC | Molecular Formula & Structure. |
|------------|---------------|-------|--------------------------------|
| 1          | Oleanolic acid | (4aS,6aS,6bR,8aR,10S,12aR,12bR,14bS)-10-hydroxy-2,2,6a,6b,9,9,12a-heptamethyl 1,2,3,4,4a,5,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-icosahydropicene-4a-carboxylic acid | C30H48O3 |
4. In-Vitro Technique

Anticancer activity of all the isolated terpenes is done by in-vitro-screening. Cell lysis of all terpenes is tested in terms of percentage on Hela cell line. The selected cell line is Hela (human cervix cancer). IC$_{50}$ and % of cell lysis were calculated [22].

The in-vitro experimental results of the Oleanolic acid, limonene and linalool with the selected Hela cell line are reported in tables 4, 5 and 6 respectively.

I] In-vitro analysis of Oleanolic acid

**Table 4. Results of in-vitro culture of oleanolic acid on Hela cell line.**

| S.No. | Compound     | Concentration (µG) | O.D. at 492nm | % of cell lysis | IC$_{50}$ value |
|-------|--------------|--------------------|---------------|-----------------|-----------------|
| 1.    | Oleanolic    | 10                 | 0.351         | 75%             | 10µG            |
| 2.    | 1            | 20                 | 0.373         | >75%            |                 |
| 3.    | 1            | 30                 | 0.545         | 100%            |                 |
| 4.    | Control      | 0.143              | 0.143         | No lysis        |                 |

II] In-vitro analysis of Limonene.

**Table 5. Results of in-vitro culture of Limonene on Hela cell line.**

| S.No. | Compound     | Concentration (µG) | O.D. at 492nm | % of cell lysis |
|-------|--------------|--------------------|---------------|-----------------|
| 1.    | Limonene     |                    |               |                 |
| 2.    | 1-Methyl-4-(1-methylethenyl)-cyclohexene | C10H16 |
| 3.    | Linalool     |                    |               |                 |
| 4.    | 1,6-Octadien-3-ol, 3,7-dimethyl- | C10H18O |
III] In-vitro analysis of linalool.

**Table 6.** Results of in-vitro culture of Linalool on Hela cell line.

| S.No. | Compound | Concentration (µG) | O.D. at 492nm | %of cell lysis | IC 50 value |
|-------|----------|-------------------|---------------|----------------|-------------|
| 1     | 3        | 10                | 0.131         | No lysis       | >30 µG      |
| 2     | 3        | 20                | 0.133         | No lysis       |             |
| 3     | 3        | 30                | 0.145         | No lysis       |             |
| 4     | Control  | -                 | 0.143         | No lysis       |             |

**Table 7.** IC50 values of isolated Terpenes on selected cell line

| Compound        | IC50 value          |
|-----------------|---------------------|
| Limonene        | >30 µg              |
| Oleanolic acid  | 10 µg               |
| Linalool        | Between 10 to 20 µG |

Table 7. Data indicates that as per the literature value, experimental values of isolated compound Oleanolic acid shows better IC50 value for Hela cell line compared to Limonene and Linalool [23, 24, 25]. Hence, it is required in less quantity for anti-cancer activity. Linalool significantly decreases the risk of human cervix cancer. Linalool appears to be beneficial after exposure on selected Hela cell line. Limonene does not reduce the risk of human cervix cancer. After exposure it is not favorable for Hela cell line at particular concentration used. It may show anticancer activity if it is used with higher concentration.

5. Conclusion

It is concluded that the Oleanolic acid is good anti-cancer agent as reported from in vitro studies. Though Oleanolic acid and Linalool shows anti-cancer activity but with this experiment it cannot be concluded that these compounds inhibits CDK2. There may be the possibilities of multi-target activity including inhibition of CDK2 [26]. The complete work anticipates that the selected plant species from Vidarbha region (Maharashtra) of India shows anti-cancer property due to the presence of terpene based natural products. In case of competitive inhibition the obtained molecules are much better than the natural inhibitors and they may support for drug like properties.

6. References

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