RESEARCH ARTICLE

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An invitro evaluation of the cytotoxicity and p53-mediated apoptotic effect of ocimum sanctum leaves hexane extract on human oral cancer cell lines

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Abstract

Background Oral cancer is one of the most common cancers and the leading cause of cancer deaths worldwide. Florae have been inspiration of salutary sources for curing ailments since ages. Hence, the contemporary investigation was aimed at evaluating the anti-proliferative activity of extract of Ocimum sanctum leaves on oral cancer KB mouth cell line.

Methods Extract preparation using soxhlet apparatus, DPPH assay for antioxidant activity, Anticancer activity and western blotting for p53 expression.

Results The obtained results showed that the methanol and hexane extracts of Ocimum sanctum (Tulsi) showed better activity in DPPH assay when compared with the rest. In anticancer activity against KB cell line and cytotoxicity activity in L132 cell line which was carried out by MTT assay; Ocimum sanctum (Tulsi) Hexane extract showed IC50 value of 74.39 for KB cell line and IC50 value of 431.39 for L132 cell line. The expression of p53 gene in KB cell line showed increased level of p53 denotes its upregulation has a cascade effect on regulation of apoptosis.

Conclusion Thus substantiating the claim that it has evidenced anti oral cancer potential, which is promising but needs additional wet lab validations and characterization to isolate the phyto molecule and qualify it as a potential lead.This promising lead could be further taken up for bio activity studies of the purified phytomolecules from the plant for in vivo testing as a potential safe herbal based alternative therapeutics for oral cancer.

Key words anti-proliferative activity, ocimum sanctum, KB mouth cell line, phytomolecules

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Introduction

Oral cancer is one of the most common cancers and the leading cause of cancer deaths worldwide. The probability of being determined to have an invasive form of cancer is presently anticipated to be as high as 40% as indicated by ongoing reports [1]. In India, the number of people living with cancer is around 2.25 million and 70 % of people died because of cancer in low and middle income countries in 2018 [2]. Oral malignancy is the 6th most normal malignant growth impelling mankind, which additionally benevolences with low rate of survival [3]. More than 90% of oral malignancies are histopathogically squamous cell carcinomas [4]. India has perhaps the most significant prevalence of oral cancer; every year around 30% people have been recognized with oral cancer because of the frequent use of tobacco, smoking and alcohol intake [5]. Other than this poor nutrition, human papilloma virus infection, genetic factors, and oral hygiene will be a threat to the cause of oral cancer [6].Current treatments incorporate chemotherapy, radiotherapy and chemically derived drugs, Pallative Treatment and Surgery. Drugs which are used most often in chemotherapy are Cisplatin [7], Carboplatin [8], 5-Fluorourasil (5-FU) [9], Paclitaxel (Taxol) [10], Docetaxel (Taxotere) [11], Hydroxyurea [12], Methotrexate [13], Bleomycin [14] and Capecitabine [15]. In any case, these treatments have slumped in numerous perspectives making human life despondent and dwindling the life expectancy of patients. Therefore, the need of great importance is to create treatment modalities by utilizing plants substances which darken intense reactions and act as powerful therapeutic agents [16]. Herbs and many plant products are evaluated as the important source of medicine because of their antiseptic properties [17]. In many developing countries herbal medicine have been utilized as an essential source of clinical treatment [18]. Various plant species are identified which possess anticancer properties that are used for the treatment and prevention of cancer development in herbal medicine in developing countries [19-20]. Compounds which have been extracted and identified for their anticancer properties include Polyphenols like flavonoids, tannins, curcumin, resveratrol, Brassinosteroids and gallacatechins are considered to be anticancer compounds[21]. Some examples of Plant-derived drugs in research and clinical trials are Sulphoraphane [22], Paclitaxel (Taxol) [10], Epipodophyllotoxin [23], Vincristine, Vinblastine, Vinorelbine, Vindesine, Vinflunine [24], Pomiferin [25], Epigallacotechin-3- gallate [26], Combretastatin A-4 phosphate [27], Roscovitine, Flavopiridol [28] and Noscapine [29]. However, these naturally derived compounds from plants are commonly more endured and non-poisonous to normal human cells [30].These drugs fall under four classes with the following activities; methytransferase inhibitors, antioxidants, histone deacetylases (HDAC) inhibitors and mitotic disruptors [31]. Based on the published literature survey and traditional folklore knowledge four plants have been chosen for the anticancer studies. They are Azadirachta indica (Neem), Mangifera indica (Mango), Ocimum sanctum (Tulsi), Carica papaya (Papaya), and Senna auriculate (Avaram Senna). Hence in the present study, is used to evaluate the effectiveness of anticancer properties of these five plants on oral cancer cell line (KB) and their expression on P53 gene and possibly exerts anticancer effects by blocking cell cycle entry into the G1 phase in oral cancer cells.

Materials and methods

Figure 1. Crude extracts of five different plant leaves (Azadirachta indica (Neem), Mangifera indica (Mango), Ocimum sanctum (Tulsi), Carica papaya (Papaya), and Senna auriculate (Avaram).

Collection of plants

Leaves of Azadirachta indica (Neem), Mangifera indica (Mango), Ocimum sanctum (Tulsi), Carica papaya (Papaya), and Senna auriculate (Avaram) were collected from Perambur, Chennai surrounding area and validated by a Botanist. Samples were washed well with running tap water followed by distilled water to remove the impurities or salt content and shade dried.

Preparation of plant extract

To extract the Phyto molecules from plants, different solvents were used. 20g of each plant material was weighed and dissolved in 200ml of solvents (1:10) methanol and hexane were used. The extraction was carried out for 24 hr at 50℃ using Soxhlet apparatus. The extracted solution was evaporated using Rotary evaporator (Bio-rad) and then dried in Hot air oven. Once dried, the plant residues were kept in sterile bottles under refrigerated conditions for further use.

Antioxidant Assay DPPH assay

The free radical scavenging capacity of the plant extract was measured based on the method delineate by Brand-Williams [32] with slight modification. It is based on electron-transfer that produces a violet solution in methanol. This free radical, which is stable at room temperature, is reduced to colorless methanol solution in the presence of an antioxidant molecule [33]. 0.1 mM DPPH (Himedia) solution was mixed with 1ml of plant extract solution of varying concentration (10, 100, 500, 1000µg/ml) tubes was incubated at room temperature for 30 minutes in dark. Then the absorbance was measured using a UV-Vis spectrophotometer at 517nm. Gallic acid (Himedia) was used as the standard [34]. The capability of scavenging the DPPH radical was calculated by using the formula:

DPPH scavenging effect/activity
(%inhibition or %scavenging) = {(A0-A1)/A0)x100}

Cell culture

The L132 and KB cell lines purchased from the NCCS Pune, India. The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100µg/ml) in a 5% CO2 at 37℃ .

Anticancer and cytotoxicity studies

Microculture tetrazolium technique colorimetric assay (MTT assay measures cell viability based on the generation of reducing equivalents in metabolic active cells [35]. The L132 and KB Cell lines (NCCS, Pune) were seeded at 5000 cells/well in 96-well plates and both were incubated for 48h at various concentrations of the sample (1, 10, 30, 100, 300, 1000 µg/ml) for 24h incubation. Then the sample was placed in a new medium containing 50µl of MTT solution (5mg/ml), to each well incubated for 4h and DMSO was added. DMSO used as a negative control for 48 h. The viable cells were determined by the absorbance at 570nm by microplate reader it was calculated by using the formula:

% Viability = {(A570 of treated cells)/(A570 of controlled cells) x100} / {% Cytotoxicity =100-%Viability}

Phytochemical analysis (qualitative)

To detect the presence of following bio molecules by standard qualitative phytochemical procedures [36-37]. Test for alkaloids using Mayer's reagent (Himedia), for tannins using ferric chloride, flavonoids using ammonia, for glycosides with chloroform and 10% ammonium solution. Saponins test done by distilled water with vigorous shaking [38], presence of terpenoid confirmed with chloroform and sulfuric acid [39]. Steroids, glycoside, phenols are also tested with standard procedures.

Thin layer chromatography

Thin layer chromatography sheets (Himedia) uses the same

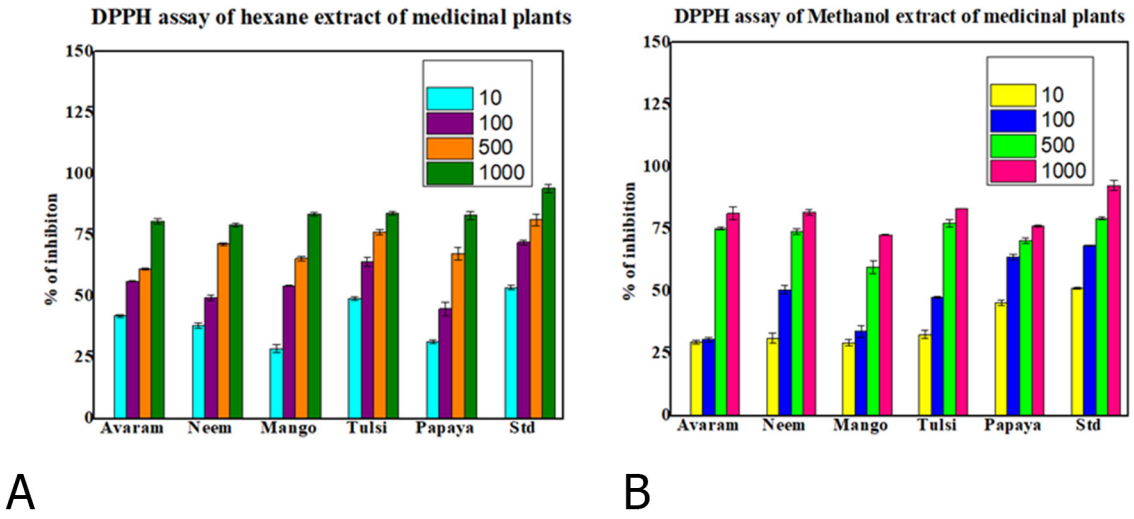

Figure 2. Percentage of inhibition of selected medicinal plant extract Azadirachta indica (Neem), Mangifera indica (Mango), Ocimum sanctum (Tulsi), Carica papaya (Papaya), and Senna auriculate (Avaram) in hexane (A)) and methanol (B) solvent on DPPH assay.

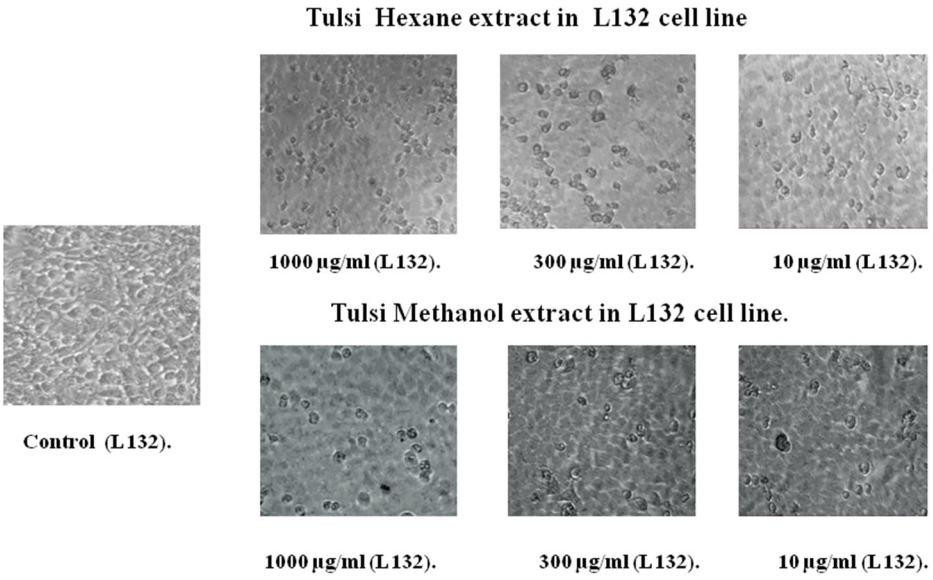

Figure 3. Ocimum sanctum (Tulsi) hexane and methanol extract treated in LI32 cell line using cell viability assay.

principles as extraction to accomplish the separation and purification of compounds. Toluene: ethyl acetate: formic acid (36:12:5) [40] is used as a mobile phase in order to separate phenolic compounds from the plant extract. The plant extract was dissolved in their particular solvent and spotted in the TLC sheet and placed in the solvent system chamber. Then they were observed for the color of the band and their fluorescence under visible light, UV-254nm and UV-366nm. For visualization iodine vapor were used. The retention factor or Rf is defined as the distance travelled by the compound divided by the distance travelled by the solvent.

Western blotting

The cells were treated with different concentration of drugs for 48h. Cells were lysed with Triple detergent lysis buffer (Electrophorosis grade) contain 50M Tris-Hcl,150 mM Nacl,0.1% SDS,100ug/ml PMSF,1ug/ml Aprorinin,1% NP-40 with pH 8.3. Add 10-20ul of sample to same amount of 2X loading buffer (1:1), mix well, and include Protein Marker boil it for 5-10 min. Quantified proteins were performed in SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes. Membrane was washed with Western Transfer buffer (10% methanol, 24mM Tris, 194mM glycine, blocked with 5-10% non-fat dried milk in

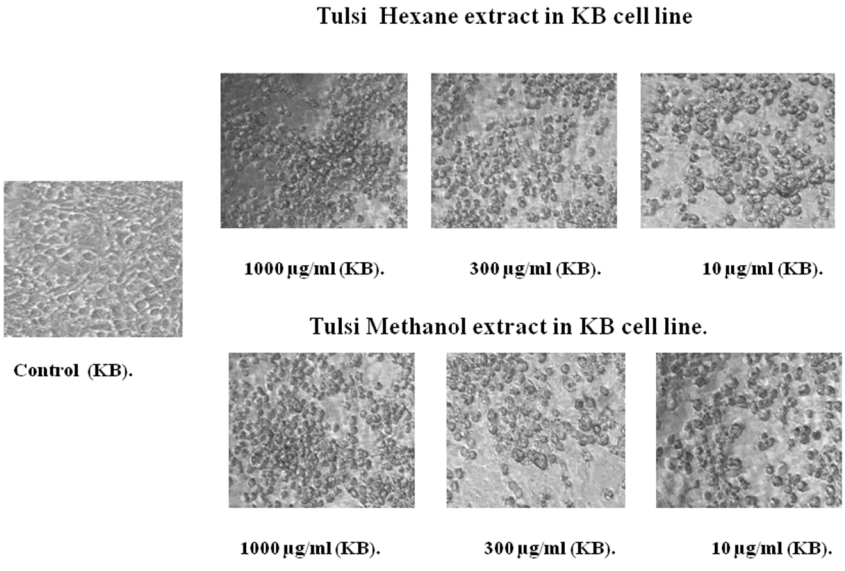

Figure 4. Ocimum sanctum (Tulsi) hexane and methanol extract treated in KB cell line MTT assay.

Table 1. Cytotoxicity effect of Ocimum sanctum (Tulsi) Hexane and methanol extract in L 132 cell line.

Concentration (µg/ml)-L132-Tulsi Hexane extract	% Live cell	Concentration (µg/ml)-L132-Tulsi Methanol extract	% Live cell
1000	67.75	1000	59.18
300	71.14	300	67.05
100	71.99	100	77.78
30	82.02	30	85.57
10	89.27	10	91.05
1	94.68	1	95.45
IC 50	431.39	IC 50	86.99

PBS for 1 hour or overnight at 4℃ with shaking. Wash in PBS with 0.1% Tween 20 for three times. The membranes then incubated with Primary Antibody in 1%BSA in PBS for 1 hour at room temperature or overnight at 4℃ and Wash in PBS with 0.1%Tween 20 for three times. Again the membranes were incubated with secondary antibody (peroxidase- conjugated goat anti-mouse IgG, etc). After Washing with PBS and 0.1%Tween 20, they were developed with DAB chromogenic detection method and scanned.

Statistical analysis

All in vitro assay data signify the mean ± standard deviation of triplicates and IC50 was calculated by using one way analysis of variances (ANOVA).

Results

Crude extract, antioxidant assay DPPH assay, and cytotoxicity test

Crude extract has been prepared from the leaves of Azadirachta indica (neem), Mangifera indica (Mango), Ocimum sanctum (Tulsi), Carica papaya (Papaya), and Senna auriculate (Avaram) in **Figure 1**. Antioxidant activity of the extracts was accessed by adopting DPPH assay. In this method the best antioxidant activity was found in Tulsi Hexane (84 %) and Tulsi Methanol (83.2%) extract. Whereas the other leaf extracts such as neem hexane 79.2 % and methanol 81.5%; papaya hexane 83.1% and methanol 76.2%; avaram hexane 80.6% and methanol 75.2%, and the values were represented in **Figure 2 (A&B)**. Based on their antioxidant property tulsi were taken to test against the oral cancer cell line. The hexane extract of O. sanctum leaves exhibited significant cytotoxic effect against L132 cell line. They were compared with control group which was untreated L132 cell line. For Cell viability test cell line was treated with both the extracts (methanol and hexane) with 6 different concentrations. The optimized IC50 values of hexane extract is 431.39 µg/ml and methanol extract are 86.99 µg/ml in **Table 1**. Further experiments have been carried out with the KB cell line to study the anticancer activity of tulsi extract (1000, 500, 100, 30, 10 and 1 µg/ml). Both the extracts exhibited promising activity with IC50 values of less than 100 µg/ml in **Table 2**.

Morphological Observation

Table 2. Anticancer activity of Ocimum sanctum (Tulsi) hexane and methanol extract in KB cell line.

Concentration (µg/ml)-L132-Tulsi Hexane extract	% Live cell	Concentration (µg/ml)-L132-Tulsi Methanol extract	% Live cell
1000	67.75	1000	59.18
300	71.14	300	67.05
100	71.99	100	77.78
30	82.02	30	85.57
10	89.27	10	91.05
1	94.68	1	95.45
IC 50	431.39	IC 50	86.99

Table 3. Phyto molecules present in Ocimum sanctum (Tulsi) Hexane extract.

Phytochemical components	Present/Absent
Flavonoids	+
Alkaloids	-
Saponins	+
Tannins	-
Phenolic compounds	+
Terpenoids	-
Steroids	+
Glycosides	-

The light microscopic observation of extracts treated with KB oral cancer cell line after 48 hours of exposure showed typical morphological features of apoptosis as aqueous concentrations increased (100,300 and 10 µg/ml). The cell death inducing ability of both extracts is determined by visual observation under inverted microscope. This is indicated by formation of fragmented apoptotic bodies which are highly condensed. In this study that the number of condensed nuclei in hexane extract of Tulsi leaves was nearly higher than that of methanol extract in KB oral cancer cells. The untreated control cell lines showed no condensed or fragmented nuclei (**Figure 3 and 4**).

Phytochemical analysis

We have concluded the presence of Flavonoids, Saponins, Phenolic compounds and Steroids in **Table 3**.

Thin layer chromatography

Thin layer chromatography was performed for Tulsi Hexane extract to separate the phenolic compounds from the crude extract. Toluene: ethyl acetate: formic acid (36:12:5) [40] is used in order to separate phenolic compounds and the image represented in **Figure**

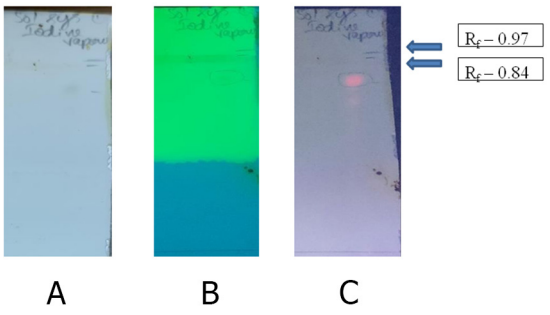

Figure 5. TLC after exposure to iodine vapor at (a) visible light, (b) UV-254 nm and (c) UV-366 nm and Rf factor is the distance travelled by the compound divided by the distance travelled by the solvent were mentioned in the right corner of the TLC sheet.

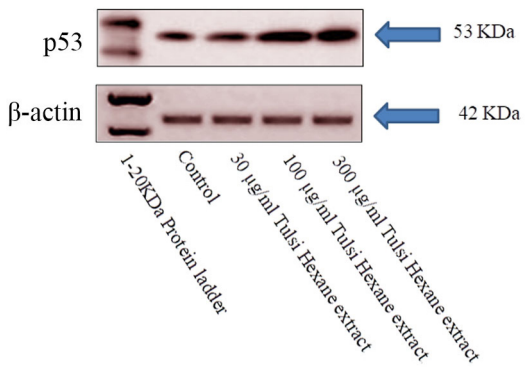

Figure 6. p53 expression in western blot, Apoptosis in oral cancer cells induced by the Hexane extract of Tulsi. Lysates of the KB cells treated with extract (30,100 and 300 µg/mL) for 24 and 48 h were subjected to an immunoblot analysis with antibodies against p53 tumor protein. Beta actin served as the loading control.

5. The Rf value of separated bands after exposure to iodine of yellow range is 0.97 and green shade 0.84 viewed in all three light rays.

p53 expression levels by Western blotting

The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading were upregulated and downregulated, respectively, in hexane tulsi extract treated cancer cells in **Figure 6**. A representative blot was shown from three independent experiments. The increase in expression of p53 can have an effect on the apoptosis of KB cell line (Oral cancer). From the result we found out that there is an increase in the expression of p53 at 100ug/ml and 300 ug/ml concentration of Tulsi Hexane extract treated cells.

Discussion

Cancer has being one of the fatal sicknesses in both developed and developing countries. The frequency of oral cancer has increased globally in recent years [41]. The Fluorouracil (5-FU) plus docetaxel treatment, with the addition of oxaliplatin chemotherapy has been widely accepted as the standard adjuvant chemotherapy for Oral squamous cell carcinoma. This study investigated the anti-cancer effects of tulsi hexane and methanol extract against KB cell line. We have used four different plant leaf based on published literature and folklore knowledge. Chemotherapeutic drugs exhibit antitumor effects by triggering apoptosis through various molecular mechanisms, has been reported in various research studies [42]. Tseng et al 2014 [43] reported that tanshinone IIA induces apoptosis in human oral cancer KB cells by mitochondria-dependent pathway. In our study result demonstrate that the hexane extract of Tulsi upregulated P53 protein expression in KB cells. Along, with our results also confirm the involvement of apoptosis in hexane extract of Tulsi induced in vitro and in vivo growth inhibition in human oral cancer cells. The best antioxidant activity was found in Tulsi Hexane and Tulsi Methanol. Leaf extract of Ocimum tenuiflorum showed high inhibition of DPPH activity of 61.11% 800µl /ml by Balaji et al., (2011) [44]. Apoptosis is an important way to maintain cellular homeostasis between cell division and cell death [45]. So, induction of apoptosis in cancer cells is one of useful strategies for anticancer drug development [46]. The cytotoxic effect was mainly because of the apoptosis is because of the presence of phytochemical compounds in the tulsi leaves. They are flavonoids, alkaloids, saponins, tannins, Phenolic compounds, terpenoids, steroids and glycosides. Other compounds such as benzene oleic acid, ethyl benzene camphene eugenol, linolenic acid, vicenin-2, citronellal, ocimarin, isorientin, circineol, myrecene, orientin, chlorogenicacid, esculectin isovitexin, gallic acid, limocene, galuteolin, rosmarinic acid, vitamin C sabinene, calcium, phosphorous and other micronutrient which may be the reason for its effectiveness against cancer cells [47-48]. The cytotoxic effect was mainly because of the apoptosis caused by the hexane extract. It is because of the presence of phytochemical compounds in the tulsi leaves. Hence Phytochemical analysis was performed for hexane extract of Ocimum sanctum find out the presence and absence of phytochemical constituents. Flavonoid synthesis in plants is induced by light colour spectrums at both high and low energy radiations. Low energy radiations are accepted by phytochrome, while high energy radiations are accepted by carotenoids, flavins, cryptochromes in addition to phytochromes. The phytochemical tests employed for alkaloids, flavonoids, glycosides, proteins, carbohydrate and tannins, Cardiac glycosides, saponins and flavonoids and terpenoids.

To gain a clearer understanding of the anti-oral cancer mechanism of hexane extract of Tulsi treated cancer cells, we investigated the effects of the tulsi extract on the expression levels and activities of intracellular signaling molecules. Cells were incubated with the indicated concentrations of hexane extract for 24 h and the whole cell lysates were analysed by western blotting using antibodies as indicated. In our study, we treated KB cells were seen under the microscope disclose the cell shrinkage and apoptotic body formation denote the characteristic of apoptosis. Whereas in the control group, the cells were regular in morphology and grew fully in patches and were confluent, rarely sloughing off. The same result was reported by Elina et al [49] in the aqueous extract of tulsi leaves. The apoptotic regulation is mainly depends upon the interbalance between Bcl-2 and Bax /p53 activity and also the other genes of their family for example Bad, Bcl-XL, Bcl-Xs and BAG1. These results suggested that the mitochondrial pathway may be involved in tulsi hexane extract induced KB cancer cell death. Mitochondria are Principal signaling centers throughout apoptosis and the loss of mitochondrial probity can be induced or hindered by numerous controllers of apoptosis where the P53 played vital role [50].

Conclusion

In this study five medicinal plant species which are commonly available in India were selected based on published literature and folklore knowledge. Tulsi leaf powders were used for extraction using two solvents. These extracts were then checked for its antioxidant property using DPPH assay and the results showed that Tulsi extracts had good antioxidant property. MTT assay were performed in KB cell line (Oral cancer cell line) and L132 cell line in order to check the anticancer and cytotoxic property and the results showed that Tulsi Hexane extract showed the best activity. Finally western blot was done to find whether the extract is having any effect in the expression of p53 gene and it showed upregulation in expression of p53. This promising lead could be taken up for bio activity studies of the purified phytomolecules from the plant for invivo testing as a potential safe herbal based alternative therapeutics for oral cancer.

Ethical policy

This article does not contain any studies with human participants or animals performed by any of the authors.

Author contributions

VRN carried out the entire lab work assays for the manuscript; JJ prepared draft document and invigilation for the manuscript; MSA carried out the extract preparation and antioxidant assay for the manuscript and VRK carried out correction and invigilation for the manuscript.

Competing interests

All authors declare no competing interests.

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