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Tinospora cordifolia (Thunb.) Miers (Giloy) inhibits oral cancer cells in a dose-dependent manner by inducing apoptosis and attenuating epithelial-mesenchymal transition



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ABSTRACT

Background: **Tinospora cordifolia** (Thunb.) Miers (Giloy) has been applied successfully as an antiinflammatory, anti-diabetic, and even as an anti-cancer agent. Yet, to date, the application of Giloy has not been explored concerning oral cancer.

Objectives: To assess the effect of *T cordifolia* (Thunb.) Miers (Giloy) extract (TcE) on an oral cancer cell line.

Methods: AW13516 (oral cancer cell line) cells were treated with the prepared aqueous extract of TCE for 24 h at various concentrations ranging between 5 µg/ml and 100 µg/ml and compared with control (cells without treatment). Thee effect of the extracts on apoptosis was assessed by through Annexin V flow cytometry assay and Luminometry based assessment of Caspase 8, 9 and caspase 3/7 activity. RNA was isolated from treated cells and gene expression of selected metastatic genes (MMP1, MMP10, and CXCL8); epithelial-mesenchymal stem cell genes (TWIST1, SNAIL, ZEB1, Oct4) and stemness related genses (Nanog, Sox2) were analyzed by using a quantitative real-time PCR system. The experiments were performed in triplicates.

Results: Aqueous extract of TcE was found to induce apoptosis inducer in AW13516 cells in a concentration-dependent manner and was potent even at a low concentration of 5 μ g/ml. The apoptosis induction was confirmed with the caspase activity assay. Treatment of the cells with the extract for 24 h exhibited a significant decrease in the expression of EMT genes in a dose-dependent manner without an effect on the metastatic genes.

Conclusion: Aqueous extract of TcE induces apoptosis-mediated cell death in the oral cancer cell line AW13516 while attenuating its potential for epithelial mesenchymal transition.

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1. Introduction

The oral cavity is one of the most common sites to be affected with cancer (Johnson et al., 2011). Globally, oral squamous cell carcinoma (OSCC) accounts for 10.4% of all malignancies and is the eighth most common neoplasm of all cancers (Amer et al., 1985). The disease is more common in Asian countries in comparison with

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Western countries (Al-Jaber et al., 2016; Joshi et al., 2014). Tobacco and alcohol are the most common risk factors for oral cancer (Shukla and Shukla, 2012). Different forms of tobacco are consumed in different parts of the world. The most toxic component of tobacco is the alkaloid Nicotine, which not only plays a vital role in carcinogenesis but also is responsible for all the hazards associated with tobacco (Corrigall, 1999; Rose and Corrigall, 1997).

The clinical presentation of oral cancer varies from reddish to white asymptomatic lesions in the initial stage to ulcers with or without pain and rigid lumps with irregular margins. Early diagnosis and prompt treatment aids in a better prognosis. However, management of oral cancer is yet another challenge as the disease is associated with morbidity affecting esthetics and function without much change in the five-year survival rate. Development of strategies for prevention and adjunct for the current therapy is the desired need of the hour. In this regard herbs containing several phytochemicals could be explored for their chemopreventive and anticancer effects.

Tinospora cordifolia (Thunb.) Miers is also known as 'Giloy' or 'Guduchi' is a genetically diverse plant found in the Indian subcontinent. It belongs to the Menispermaceae family. It grows at high altitude and bears flowers that are greenish to yellow, found at higher altitude (Parthipan et al., 2011; Rana et al., 2012; The Ayurvedic Pharmacopoeia of India, 2001). Indian Pharmacopoeia has identified the medicinal properties of *T. cordifolia* and the same is a vital constituent of several medicinal formulations for the management of several diseases and discomfort including pyrexia, dyspepsia, syphilis, gonorrhea, diseases of the urinary tract, gout, viral hepatitis, anemia, general weakness, urinary tract infections, dermatological diseases, loss of appetite, asthma (Chintalwar et al., 1999; Gupta et al., 1967; Chopra et al., 1958; Upadhyay et al., 2010).

Thus, it has attracted the attention of researchers with its numerous biological roles. Most importantly, the active compounds of this plant contribute to its advantageousness that includes glycosides, steroids sesquiterpenoid, phenolics, polysaccharides, diterpenoid lactones, aliphatic compounds, and alkaloids (Upadhyay et al., 2010). Its various roles as an anti-inflammatory, anti-diabetic, anti-cancer, etc. have been reported (Ghosh and Saha, 2012). However, the anticancer effect on oral cancer has not been reported so far. With the available data, we aimed to explore the anti-cancer effect of *Tinospora cordifolia* (Thunb.) Miers (Giloy) extract (TCE) on oral cancer cell line AW13516.

2. Materials and methods

2.1. Preparation of TcE

Commercially available finely ground *T. cordifolia* stem powder (Dabur India Ltd., India) was obtained. After weighing, the powder was subjected to soaking it in a distilled water-filled conical flask for 24 h, under continuous agitation at room temperature. The same was repeated until the supernatant water was found to be colorless. The particulate matters which were not dissolved were filtered by a syringe filter of pore size 0.22 μ m (Corning, NY, USA). The remaining filtrate was lyophilized to make dried powdered material, which was then assessed for weight. The extract (100 mg) was mixed in 100 mg/mL of water to prepare the stock solution, which was then refrigerated to be used for further analysis.

2.2. Protocol for cell culture of AW13516 cells

The oral squamous cell carcinoma cell line AW13516 was obtained from ACTREC, Tata Memorial Centre, Kharghar, Navi

Mumbai, India. The cells were further expanded in a complete medium containing Dulbecco's Modified Eagles Medium(DMEM) (Invitrogen, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) with incubation at 37 °C and 5% CO₂.

2.3. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for assessment of cell viability

The viability of the cells was assessed by MTT assay. The AW13516 cells were seeded into 96-well plates at the cell density of 5×10^3 cells per well and incubated for 24 h. Further, the adhered cells were treated with appropriate concentrations of TcE (5 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml) mixed with the complete medium (DMEM + 10% fetal bovine serum [FBS]) and incubated for 48 h. To this addition of 0.5 mg/ml MTT solution (Sigma-Aldrich Corp., St. Louis, MO, USA) was done. On completion of 4 h incubation, removal of the medium was done. To each well, the addition of 100 µl dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was made and absorbance was measured at 570 nm using a Multiskan FC spectrophotometer (Thermo Scientific, San Jose, CA, USA) (Mosmann, 1983).

2.4. Assessment of apoptosis by Annexin V-FITC/PI assay

Apoptosis detection assay was performed using Annexin V-FITC/PI (BD Pharmingen, San Diego, CA, USA) staining. The AW13516 cells were seeded into 12-well plates at the density of 1×10^5 cells per well and incubated for 24 h. Further, the cells were treated with appropriate concentrations of TcE (5 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml) mixed with the complete medium (DMEM + 10% FBS) and incubated for 48 h. The treated and untreated cells were harvested and stained with Annexin V-FITC reagent. The cells were incubated for 15 min at room temperature in the absence of light. After incubation, the PI reagent was introduced to the cells and immediately assayed by flow cytometry (Attune NxT, Thermo Fisher Scientific, Waltham, MA, USA). At least 10,000 events were acquired for each group. The percentage of cells undergoing apoptosis was calculated.

2.5. Determination of Caspase activity

Caspase-3 and -7, Caspase-8, and Caspase-9 activities were determined by using the Caspase-Glo assay kits (Promega, Madison, WI, USA) as per the manufacturer's instructions. The AW13516 cells were seeded into 12-well plates at the density of 1×10^5 cells per well and incubated for 24 h. Further, the cells were treated with appropriate concentrations of TcE (5 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml) mixed with the complete medium (DMEM + 10% FBS) and incubated for 48 h. Luminescence was detected using the GloMax Multi Luminescence Multimode Reader (Promega, Madison, WI, USA).

2.6. Detection of mitochondrial potential by flow cytometry

To detect mitochondrial membrane potential in treated and untreated cells, a mitochondrial potential kit was used (Sigma Aldrich, St. Louis, MO, USA). The AW13516 cells were seeded into 12-well plates at the cell density of 1×10^6 cells per well and incubated for 24 h. Further, the adhered cells were treated with appropriate concentrations of TcE (5 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ ml, and 100 µg/ml) mixed with the complete medium (DMEM + 10% FBS) and incubated for 48 h. The cells were stained with 10 µg/ml 1,1',3,3,3'.4-hexamethylindodicarbo-cyanine iodide (DiIC1). For the control tube untreated cells were used, and negative control cells were treated with 50 mM Carbonyl cyanide 3chlorophenylhydrazone (CCCP) following which it was incubated

Table 1

List of primers.

Gene	Forward Primer	Reverse Primer
TWIST1	5'-GCC AGG TAC ATC GAC TTC CTC T-3'	5'-TCC ATC CTC CAG ACC GAG AAG G-3'
SNAIL	5'-TGC CCT CAA GAT GCA CAT CCG A-3'	5'-GGG ACA GGA GAA GGG CTT CTC-3'
ZEB1	5'-GGC ATA CAC CTA CTC AAC TAC GG-3'	5'-TGG GCG GTG TAG AAT CAG AGT C-3'
MMP1	5'-ATG AAG CAG CCC AGA TGT GGA G-3'	5'-TGG TCC ACA TCT GCT CTT GGC A-3'
MMP10	5'-TCC AGG CTG TAT GAA GGA GAG G-3'	5'-GGT AGG CAT GAG CCA AAC TGT G-3'
IL8 (CXCL8)	5'-GAG AGT GAT TGA GAG TGG ACC AC-3'	5'-CAC AAC CCT CTG CAC CCA GTT T-3'
OCT4	5'-GTG GAG GAA GCT GAC AAC AA-3'	5'-ATT CTC CAG GTT GCC TCT CA-3'
SOX2	5'-CCA GCA GAC TTC ACA TGT CC-3'	5'-ACA TGT GTG AGA GGG GCA GT-3'
NANOG	5'-TTT GTG GGC CTG AAG AAA ACT-3'	5'-AGG GCT GTC CTG AAT AAG CAG-3'
GAPDH	5'-GTC TCC TCT GAC TTC AAC AGC G-3'	5'-ACC ACC CTG TTG CTG TAG CCA A-3'



Fig. 1. Cell viability by MTT assay, mitochondrial potential by flow cytometry, and analysis of apoptosis by annexin-V/Pl assay. (A) MTT assay was performed to assess the viability of AW13516 cells treated with various concentrations of TcE (5 μ g/ml, 10 μ g/ml, 25 μ g/ml, 50 μ g/ml, and 100 μ g/ml). (B) Mitochondrial potential (MFI) was evaluated by flow cytometry in the AW13516 cells treated with various concentrations of TcE (5 μ g/ml, 10 μ g/ml, 10 μ g/ml, 25 μ g/ml, 50 μ g/ml, 50 μ g/ml, and 100 μ g/ml). (C-I) Percentage apoptosis was calculated in the AW13516 cells treated with various concentrations of TcE (5 μ g/ml, 10 μ g/ml, 25 μ g/ml, 50 μ g/ml, and 100 μ g/ml) after performing the annexin-V/Pl assay on flow cytometry. ns not significant, *p < 0.05, **p < 0.001. TcE: T. cordifolia aqueous extract, MFI: median fluorescence intensity.

at 37 °C at 5% CO2 for 15–30 min. Post incubation, phosphate buffered saline (PBS) was used to wash the cells before they were analyzed using a flow cytometer. At least 10,000 events were acquired for each group. The data was calculated as median fluorescence intensity (MFI).

2.7. Real-time qPCR for gene expression analysis

The AW13516 cells were seeded into 12-well plates at the cell density of 1×10^5 cells per well and incubated for 24 h. For the assessment of epithelial to mesenchymal transition (EMT). EMT was induced in AW13516 cells by treating the cells with 10 ng/ mL human TGF-B1 recombinant (R&D System, Minneapolis, MN, USA) for 2 h. Further, the adhered cells were treated with appropriate concentrations of TcE (5 μ g, 10 μ g, 25 μ g, 50 μ g, and 100 μ g) mixed with the complete medium (DMEM + 10% FBS) and incubated for 48 h. After the incubation, the cells were trypsinized from the plates and the extraction of total RNA was done with Genelet purification columns (Invitrogen, Thermo Scientific, Lithuania). 1 µg of total RNA was transcribed reversely by using cDNA synthesis (High Capacity, Applied Biosystems, Carlsbad, CA, USA). Quantitative analyses of gene expressions were done by using the SYBRGreen PCR master mix (Applied Biosystems, Austin, TX, USA) on QuantStudio 5 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Expressions of the target genes (TWIST1, SNAIL, ZEB1, MMP1, MMP10, IL8/CXCL8, Oct4, Nanog, Sox2) were normalized to GAPDH by the $\Delta\Delta$ Ct technique. The quantification of the data obtained from RT-PCR was done by calculating $2^{-\Delta\Delta$ Ct} values. The list of the primers (IDT, Coralville, IA, USA) has been given in the Table 1.

2.8. Statistical analysis

Each experiment was replicated in triplicates. The results were shown as the mean \pm standard deviation of the values from the three independent experimental values. The data were analyzed by using unpaired *t*-test (two-tailed) on GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA) for each cytokine, and the p < 0.05 was considered as significant (ns not significant, *p < 0.05, and **p < 0.01).

3. Results

3.1. TcE shows the concentration-dependent effect on viability, mitochondrial potential, and apoptosis in AW13516 cells

The treatment with increasing concentrations of TcE showed a significantly decreased viability in the AW13516 cells in a concentration-dependent manner depicted by the MTT assay (Fig. 1A). Annexin V assay by flow cytometry revealed that the extract was found to induce apoptosis in the AW13516 cells and was potent even at a low concentration of 5 μ g/ml. The percentage



Fig. 2. Caspase activity by luminometry. (A-C) Caspase-3/7, Caspase-8, and Caspase-9 activities were examined by luminometric assays in the AW13516 cells treated with various concentrations of TcE. ns not significant, *p < 0.05, **p < 0.001. TcE: T. cordifolia aqueous extract.



Fig. 3. Analysis of EMT-related gene expression by quantitative RT-PCR. (A-C) EMT was induced by TGF-β1 in the AW13516 cells and comparative gene expression analysis was carried out in the AW13516 cells treated with various concentrations of TcE for EMT-related transcription factors by RT-qPCR for TWIST1, SNAIL, and ZEB1. n.s. not significant, *p < 0.05, **p < 0.001. EMT: epithelial-mesenchymal transition, TcE: T. cordifolia aqueous extract, TWIST1: Twist family BHLH transcription factor 1, SNAIL: Snail family transcriptional repressor 1, ZEB1: Zinc finger E-box-binding homeobox 1.

of apoptotic cell death significantly increased with an increase in the concentration of the TcE (Figs. 1C and 2I). The apoptosis induction was via mitochondrial pathway depicted by a decrease in mitochondrial membrane potential induced by the extract on the cells in a concentration-dependent manner (Fig. 1B).

3.2. TcE augmented the caspase activity in the AW13516 cells in a concentration-dependent manner

Caspase activity, which is characteristic of apoptotic cell death, was assessed to confirm the results of the Annexin V assay. The cells on treatment with TcE showed increased Caspase-3 and -7, Caspase-8, and Caspase-9 activities with an increase in concentration (Fig. 2A–C).

3.3. TcE attenuates induced epithelial to mesenchymal transition (EMT) in AW13516 cells

The AW13516 cells were treated with TGF- β 1 to induce EMT and subsequently, the same cells were treated with TcE. The gene expression of EMT-related transcription factors showed decreased expression with an increase in concentrations of TcE. The expression levels of TWIST1 decreased significantly with the treated cells, with the highest decrease in expression of TWIST1at 100 µg/mL of TcE (Fig. 3A). The same trend was observed in the expression levels of SNAIL except at 5 µg/mL TcE where a significant effect was absent (Fig. 3B). Whereas ZEB1 expression levels were found to be significantly decreased in only 25 µg/mL, 50 µg/mL, and 100 µg/mL TcE treated AW13516 cells (Fig. 4C). 5 µg/mL and



Fig. 4. Analysis of metastasis-related gene expression by quantitative RT-PCR. (A-C) Comparative gene expression analysis was carried out in the AW13516 cells treated with various concentrations of TcE for metastasis-related transcription factors by RT-qPCR for MMP1, MMP10, and CXCL8. n.s. not significant, *p < 0.05, **p < 0.001. TcE: T. cordifolia aqueous extract, MMP1: Matrix metallopeptidase 1, MMP10: Matrix metallopeptidase 10, CXCL8: C-X-C motif chemokine ligand 8.

10 $\mu g/mL$ TcE did not exert any significant effect on the expression levels of ZEB1.

3.4. TcE did not affect the expression levels of metastasis-related genes in AW13516 cells

MMP1, MMP10, and CXCL8 are highly implicated in the metastatic activity of oral squamous cell carcinoma. None of the concentrations of TcE at all concentrations exhibited no significant difference in the expression levels of MMP1, MMP10, and CXCL8 (Figs. 4A and 5C).

3.5. Treatment with TcE reduced the gene expression of stemness related genes slightly in a nonsignificant manner

Oct-4, Sox2, and Nanog are the factors that maintain the stemness of the cells including cancer stem cells. When analyzed for the expression changes due to TcE treatment, it was observed that higher concentrations of TcE showed decreased expression levels of only OCT4 and NANOG (Fig. 5A & C) but no concentration of TcE affected the expression level of SOX2 (Fig. 5B).

The raw files generated from the study are presented as supplementary files and data in brief.

4. Discussion

OSCC is one of the most common malignancies of the head and neck region globally (Baig et al., 2018; Desideri et al., 2018; Printz, 2016). Although the disease is multifactorial in etiology, tobacco has been reported to be the major risk factor of OSCC (Alsanosy,



Fig. 5. Analysis of stemness-related gene expression by quantitative RT-PCR. (A-C) Comparative gene expression analysis was carried out in the AW13516 cells treated with various concentrations of TCE for stemness-related transcription factors by RT-qPCR for OCT4, SOX2, and NANOG. n.s. not significant, *p < 0.05, **p < 0.001. TCE: T. cordifolia aqueous extract, OCT4: Octamer-binding transcription factor 4, SOX2: SRY (sex-determining region Y)-box 2, NANOG: Homeobox protein NANOG.

2014; Johnson, 2001; Samman, 1998; Walsh and Epstein, 2000; Warnakulasuriya, 2004). Recently several awareness programs on the health hazards of tobacco are being conducted yet nicotine addiction and its consequences have not decreased (Weintraub and Hamilton, 2002). The increased incidence rate and a static five year survival rate of oral cancer, has attracted interest in exploring the medicinal properties of naturally occurring plant and plant products for their anticancer effects. A thorough understanding of the molecular mechanisms of the disease and pharmagonostic effect of naturally occurring plant and plant products is necessary to develop a therapeutic adjunct for oral cancer. Herbs contain several vital phytochemicals that possess medicinal properties which have been explored for the management of various illness and ailments.

In the present study, *T. cordifolia* was selected due to fact that this shrub is indigenous to India and its easy availability (Virginia and Premila, 2006). It has found its medicinal use for the management of various diseases in traditional medicine (Kumar et al., 2020). It has also been referred to as Guduchi (protector of diseases) in the most ancient books such as Sushruta Samhita and - Charaka Samhita. The active compounds of this plant that contribute to the medicinal properties include glycosides, steroids sesquiterpenoid, phenolics, polysaccharides, diterpenoid lactones, aliphatic compounds and alkaloids (Upadhyay et al., 2010). Its various roles as an anti-inflammatory, anti-diabetic, anti-cancer, etc. have been reported (Ghosh and Saha, 2012). However, the effect of the extract on oral cancer has not been reported to date. Hence the present study was conducted to explore the effect of TcE on an oral cancer cell line.

The MTT assay revealed induction of cell death by TcE in a concentration-dependent manner. The results are concurrent with the findings of Rao et al and Palmieri et al who have reported decreased cell viability on different cell lines (Palmieri et al., 2019; Rao and Rao, 2010). The extract exhibited anticancer by effects by induction of apoptosis depicted by Annexin V stain and confirmed by Caspase activity assays. The apoptosis induction was via alteration of mitochondrial membrane potential in a concentration-dependent manner. The findings are concurrent with the findings reported by Mishra et al who have reported similar effects on the glioblastoma cell line (Mishra and Kaur, 2013).

The extract was known to modulate genes responsible for epithelial-mesenchymal transition which is one of the most important pathways of carcinogenesis. The results are concurrent with the findings of Palmieri et al who have reported a similar effect of the extract on epithelial-mesenchymal transition on the human colon adenocarcinoma (HCA-7) cell line (Palmieri et al., 2019). However, the extract did not exert an effect on the genes involved in metastasis. The results are contrary to the findings reported by Leyon et al. in their study they observed a significant reduction of the metastatic ability of B16-F10 melanoma cells when polysaccharide fraction was injected intraperitoneally in mice previously injected with the melanoma cells (Leyon and Kuttan, 2004). This could be attributed to the fact the preparation of the plant extract was different that could affect the phytochemical constituent and medicinal property. Also, the variation in the property of the cell line studied could have led to contrary results.

Cancer stem cells are the population within a tumor responsible for metastasis, drug resistance, and immune evasion, many transcription factors, and proteins involved in stemness maintenance in normal stem cells are found to be playing role in cancer stem cells. Some of them are Oct4, Sox2, Nanog, and CD34, etc. In the current study when the gene expression was compared between treated and untreated cells, it was evident that treatment with TcE decreased the expression of all three important transcription Oct4, Sox2, and Nanog. Even the values have decreased the expression was not much significant in both treated and untreated cells. this can be because cancer stem cell frequencies are very less in the overall cell line population. This effect on cancer stem cells should be repeated in the future after sorting the cancer stem cell population from the cell line. The anticancer activity of TcE could be attributed to berberine (BBR) that has been reported for its medicinal properties such as diabetes, diarrhea, gastroenteritis, dyslipidemia, cardiac diseases, inflammatory diseases, and obesity by various authors (Chen et al., 2014; Lahiri and Dutta, 1967; Liu et al., 2015; Sun et al., 1988). Moreover, the anticancer activity of this compound has been reported in various cell lines (Tillhon et al., 2012). BBR has been reported to cause apoptosis via activation of Janus Kinase, p38 mitogen-activated protein kinase pathway, suppression Wnt/β-catenin signaling pathway, and inhibition of mitosis through G1/S and G2/M cell cycle arrest induction concentration (Albring et al., 2013; Chidambara Murthy et al., 2012).

5. Conclusion

TcE induced apoptosis in AW13516 cells in a concentrationdependent manner. Even at a low concentration of 5 μ g/ml, apoptosis was induced. Also, a 24 h treatment with Giloy induced a significant reduction in the expression of EMT genes in a dosedependent manner, although no effect was noted on the metastatic genes. Further studies could be carried out to explore the in-vivo anticancer effects of **T. cordifolia** and berberine on oral cancer. The extract could also be explored for the cancer-preventive activity and management of potentially malignant disorders.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.04.056.

References

- Al-Jaber, A., Al-Nasser, L., El-Metwally, A., 2016. Epidemiology of oral cancer in Arab countries. Saudi Med. J. 37, 249–255. https://doi.org/10.15537/ smj.2016.3.11388.
- Albring, K.F., Weidemüller, J., Mittag, S., Weiske, J., Friedrich, K., Geroni, M.C., Lombardi, P., Huber, O., 2013. Berberine acts as a natural inhibitor of Wnt/βcatenin signaling-Identification of more active 13-arylalkyl derivatives. BioFactors 39, 652–662. https://doi.org/10.1002/biof.1133.
- Alsanosy, R.M., 2014. Smokeless Tobacco (Shammah) in Saudi Arabia: A Review of its Pattern of Use, Prevalence, and Potential Role in Oral Cancer. Asian Pacific J. Cancer Prev. 15, 6477–6483. https://doi.org/10.7314/APJCP.2014.15.16.6477.
- Amer, M., Bull, C.A., Daouk, M.N., McArthur, P.D., Lundmark, G.J., El Senoussi, M., 1985. Shamma Usage and Oral Cancer in Saudi Arabia. Ann. Saudi Med. 5, 135– 140. https://doi.org/10.5144/0256-4947.1985.135.
- Baig, S., Rubab, Z., Farooq, W., 2018. Molecular Pathogenesis of Chewable Tobacco. J. Coll. Physicians Surg. Pakistan 28, 381–385. https://doi.org/10.29271/ jcpsp.2018.05.381.
- Chen, C., Yu, Z., Li, Y., Fichna, J., Storr, M., 2014. Effects of Berberine in the Gastrointestinal Tract – A Review of Actions and Therapeutic Implications. Am. J. Chin. Med. 42, 1053–1070. https://doi.org/10.1142/S0192415X14500669.
- Chidambara Murthy, K.N., Jayaprakasha, G.K., Patil, B.S., 2012. The natural alkaloid berberine targets multiple pathways to induce cell death in cultured human colon cancer cells. Eur. J. Pharmacol. 688, 14–21. https://doi.org/10.1016/j. ejphar.2012.05.004.
- Chintalwar, G., Jain, A., Sipahimalani, A., Banerji, A., Sumariwalla, P., Ramakrishnan, R., Sainis, K., 1999. An immunologically active arabinogalactan from Tinospora cordifolia. Phytochemistry 52, 1089–1093. https://doi.org/10.1016/S0031-9422 (99)00386-6.
- Chopra, R.N., Chopra, I.C., Handa, K.L., Kapur, L.D., 1958. Chopra's Indigenous Drugs of India. B.K. Dhur of Academic Publishers, Kolkatta, India.
- Corrigall, W., 1999. Nicotine self-administration in animals as a dependence model. Nicotine Tob. Res. 1, 11–20. https://doi.org/10.1080/14622299050011121.
- Desideri, D., Roselli, C., Fagiolino, I., Meli, M.A., 2018. Toxic Elements in Human Saliva of Smokeless Tobacco Users. J. Anal. Toxicol. 42, 417–424. https://doi.org/ 10.1093/jat/bky019.
- Ghosh, S., Saha, S., 2012. Tinospora cordifolia: One plant, many roles. Anc. Sci. Life 31, 151. https://doi.org/10.4103/0257-7941.107344.
- Gupta, S.S., Verma, S.C., Garg, V.P., Rai, M., 1967. Anti-diabetic effects of Tinospora cardifolia. I. Effect on fasting blood sugar level, glucose tolerance and adrenaline induced hyperglycaemia. Indian J. Med. Res. 55, 733–745.
- Johnson, N., 2001. Tobacco use and oral cancer: a global perspective. J. Dent. Educ. 65, 328–339.
- Johnson, N.W., Jayasekara, P., Amarasinghe, A.A.H.K., 2011. Squamous cell carcinoma and precursor lesions of the oral cavity: epidemiology and aetiology. Periodontol. 2000 (57), 19–37. https://doi.org/10.1111/j.1600-0757.2011.00401.x.
- Joshi, P., Dutta, S., Chaturvedi, P., Nair, S., 2014. Head and Neck Cancers in Developing Countries. Rambam Maimonides Med. J. 5,. https://doi.org/10.5041/ RMMJ.10143 e0009.

- Kumar, P., Kamle, M., Mahato, D.K., Bora, H., Sharma, B., Rasane, P., Bajpai, V.K., 2020. Tinospora cordifolia (Giloy): Phytochemistry, Ethnopharmacology, Clinical Application and Conservation Strategies. Curr. Pharm. Biotechnol. 21, 1165–1175. https://doi.org/10.2174/1389201021666200430114547.
- Lahiri, S.C., Dutta, N.K., 1967. Berberine and chloramphenicol in the treatment of cholera and severe diarrhoea. J. Indian Med. Assoc. 48, 1–11.
- Leyon, P., Kuttan, G., 2004. Inhibitory effect of a polysaccharide from Tinospora cordifolia on experimental metastasis. J. Ethnopharmacol. 90, 233–237. https:// doi.org/10.1016/j.jep.2003.09.046.
- Liu, C., Wang, Z., Song, Y., Wu, D., Zheng, X., Li, P., Jin, J., Xu, N., Li, L., 2015. Effects of Berberine on Amelioration of Hyperglycemia and Oxidative Stress in High Glucose and High Fat Diet-Induced Diabetic Hamsters In Vivo. Biomed Res. Int. 2015, 1–9. https://doi.org/10.1155/2015/313808.
- Mishra, R., Kaur, G., 2013. Aqueous Ethanolic Extract of Tinospora cordifolia as a Potential Candidate for Differentiation Based Therapy of Glioblastomas. PLoS One 8, https://doi.org/10.1371/journal.pone.0078764 e78764.
- Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63. https://doi.org/10.1016/0022-1759(83)90303-4.
- Palmieri, A., Scapoli, L., Iapichino, A., Mercolini, L., Mandrone, M., Poli, F., Gianni, A. B., Baserga, C., Martinelli, M., 2019. Berberine and Tinospora cordifolia exert a potential anticancer effect on colon cancer cells by acting on specific pathways. Int. J. Immunopathol. Pharmacol. 33, 205873841985556. https://doi.org/ 10.1177/2058738419855567.
- Parthipan, M., Aravindhan, V., Rajendran, A., 2011. Medico-botanical study of Yercaud hills in the eastern Ghats of Tamil Nadu. India. Anc. Sci. Life 30, 104– 109.
- Printz, C., 2016. Users of smokeless tobacco had higher levels of exposure to nicotine, carcinogens than cigarette smokers. Cancer 122, 987–987. https://doi. org/10.1002/cncr.29949.
- Rana, Vi, Thakur, K., Sood, R., Sharma, V., Sharma, T.R., 2012. Genetic diversity analysis of Tinospora cordifolia germplasm collected from northwestern Himalayan region of India. J. Genet. 91, 99–103. https://doi.org/10.1007/ s12041-012-0137-7.
- Rao, S.K., Rao, P.S., 2010. Alteration in the Radiosensitivity of HeLa Cells by Dichloromethane Extract of Guduchi (Tinospora cordifolia). Integr. Cancer Ther. 9, 378–384. https://doi.org/10.1177/1534735410387598.
- Rose, J.E., Corrigall, W.A., 1997. Nicotine self-administration in animals and humans: similarities and differences. Psychopharmacology (Berl). 130, 28–40. https://doi.org/10.1007/s002130050209.
- Samman, M., 1998. Mint prevents shamma-induced carcinogenesis in hamster cheek pouch. Carcinogenesis 19, 1795–1801. https://doi.org/10.1093/carcin/ 19.10.1795.
- Shukla, Sumit, Shukla, Satish, 2012. Oral Cancer–Curse, Cure and Challenge. Indian. J. Surg. 74, 437–439. https://doi.org/10.1007/s12262-012-0769-0.
- Sun, D., Abraham, S.N., Beachey, E.H., 1988. Influence of berberine sulfate on synthesis and expression of Pap fimbrial adhesin in uropathogenic Escherichia coli. Antimicrob. Agents Chemother. 32, 1274–1277. https://doi.org/10.1128/ AAC.32.8.1274.
- The Ayurvedic Pharmacopoeia of India, 1st ed, 2001. Department Of AYUSH, Ministry of Health and FW, New Delhi.
- Tillhon, M., Guamán Ortiz, L.M., Lombardi, P., Scovassi, A.I., 2012. Berberine: New perspectives for old remedies. Biochem. Pharmacol. 84, 1260–1267. https://doi. org/10.1016/j.bcp.2012.07.018.
- Upadhyay, A., Kumar, K., Kumar, A., Mishra, H., 2010. Tinospora cordifolia (Willd.) Hook. f. and Thoms. (Guduchi) - validation of the Ayurvedic pharmacology through experimental and clinical studies. Int. J. Ayurveda Res. 1, 112. https://doi.org/10.4103/0974-7788.64405.
- Virginia, M.T., Premila, M., 2006. Ayurvedic Herbs: A Clinical Guide to the Healing Plants of Traditional. Haworth Press, New York.
- Walsh, P.M., Epstein, J.B., 2000. The oral effects of smokeless tobacco. J. Can. Dent. Assoc. 66, 22–25.
- Warnakulasuriya, S., 2004. Smokeless tobacco and oral cancer. Oral Dis. 10, 1–4. https://doi.org/10.1046/j.1354-523X.2003.00975.x.
 Weintraub, J.M., Hamilton, W.L., 2002. Trends in prevalence of current smoking,
- Weintraub, J.M., Hamilton, W.L., 2002. Trends in prevalence of current smoking, Massachusetts and states without tobacco control programmes, 1990 to 1999. Tob. Control 11 Suppl 2, ii8-13. https://doi.org/10.1136/tc.11.suppl_2.ii8.