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Pongamol from the seeds of *Tephrosia purpurea* exert anti-cancer activity against cervical cancer cell line

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Tephrosia purpurea belongs to the family Fabaceae, is used for the treatment of inflammation, diabetes, cancer, chronic fever, boils, gingivitis etc. In the present study, three compounds (TP-1, TP-3 and TP-5) were isolated from ethyl acetate extract of the seeds of *T. purpurea* (EETP). Quantity of TP-3 (6.05%) in EETP was determined by HPLC. *In-vitro* anti-cancer activity of EETP and isolated compound TP-3 on SiHa cells as well as PBMCs (peripheral blood mononuclear cells) were evaluated by MTT (3-(4,5- di methyl thiazol -2-yl)-2,5-di phenyl tetrazolium bromide) assay. On the basis of spectroscopic analysis and physical properties, structure of isolated compounds TP-1, TP-3 and TP-5 were characterized as teclenone, pongamol and *β*-sitosterol respectively.

Isolated compound TP-3 showed cytotoxicity against SiHa cells with the IC_{50} 33.06 μgmL^{-1}. TP-3 showed significant cytotoxicity at the concentration range from 20-50 μgmL^{-1} as compared to control. Maximum cytotoxicity (72.75%) was observed at the concentration of 50 μgmL^{-1}. TP-3 showed non-significant cytotoxicity against PBMCs cells at all the concentrations except at 50 μgmL^{-1} (cytotoxicity 24.19%). EETP showed significant cytotoxicity (IC_{50} value 113.63 μgmL^{-1}) against SiHa cells at the concentration range from 75-150 μgmL^{-1} as compared to control. Maximum cytotoxicity (75.34%) was observed at the concentration of 150 μgmL^{-1}.

Keywords: MTT assay, PBMCs cells, SiHa Cells, Teclenone, *β*-sitosterol

IPC Code: Int Cl.[23]: A61K 36/00

Tephrosia purpurea (Linn.) pers. belongs to the family Fabaceae, is found in Africa, Southeast Asia, Australia, China, Sri Lanka and India. In India, *T. purpurea* is found in Uttar Pradesh, Andhra Pradesh, Haryana, Rajasthan and Tamilnadu[1]. *T. purpurea* is a perennial, herbaceous and abundantly branched or shaggy herb. Generally the height of the plant is 30-60 cm, sometime upto 1.5 m. The spreading branches are sparsely pilous or glabrous. In Koman (Northern Albania), a decoction of the drug (1 part in 10 part of water) is administered in one ounce (29.57 mL) doses to treat Bight's disease with dropsy and found to possess diuretic properties in mild degree[2]. Root of *T. purpurea* is used for the treatment of inflammation, skin disorders, elephantitis, haemmaroids, asthma, bronchitis, anaemia, dysmenorrhea, chronic fever, boils, pimples and gingivitis[3]. In India and China, seeds are used as a substitute for coffee[4]. Different category of phytochemical constituents reported from the plant *T. purpurea* are rotenoids, isoflavones, flavanones, chalcones, sterols, flavonols, flavones, terpenoids and other secondary metabolites. Seeds of the *T. purpurea* contain pongamol, isolonchocarpin, karanjin, lanceolatin B, kanjone, sitosterol, purpurin, pupuritenin and purpureamethide[5-7]. Pharmacological activities reported for the plant *T. purpurea* are hepatoprotective[8-10], anti-inflammatory, analgesic[11,12], antimicrobial[13-16], anti-ulcer[17,18], antidiabetic[19,20], anti-tuberculosis[21], antioxidant[22,23] and cytotoxic[24-26]. Three novel flavonoids having tetrahydrofuran moiety (+)-tephrorins A, tephrorin B and (+)-tephrosone were isolated from *T. purpurea* and evaluated for their

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potential chemo-preventive properties using quinone reductase induction assay[27]. A novel flavonoid having acetylcholinesterase inhibitory activity isolated from the leaves of *T. purpurea*[28].

Present study was carried out for isolation of phytochemical constituents from the seeds of *T. purpurea* and characterization of isolated compound by spectroscopic analysis. Since this plant is reported to have anti-cancer property by previous authors, an attempt has been also made to find the active phytochemical constituents responsible for the cytotoxicity of *T. purpurea*.

Materials and Methods

Chemicals and reagents

Human cervical cancer cell line (SiHa cells) was obtained from the National Centre for Cell Science, Pune, India. MTT was purchased from HiMedia laboratories Pvt. Ltds., India. All other chemical used in the present work were of analytical grade.

Plant material

Seeds of *T. purpurea* were collected during the month of September 2017, from the Rajiv Gandhi South Campus, Banaras Hindu University, Barkachha, Mirzapur, Uttar Pradesh, India. Plant specimen (whole plant) was authenticated at Botanical Survey of India, Howrah, West Bengal, India (plant identification letter: CNH/2017/Tech. II/48, speciemen No. SK-01, Dated: 17-10-2017). A voucher specimen of plant (Voucher No. PRL-08) has been deposited at Pharmacy Research Laboratoty, Rajiv Gandhi South Campus, Banaras Hindu University, Barkachha, Mirzapur, Uttar Pradesh, India for future reference.

Preparation of plant extract

Seeds of *T. purpurea* were dried and converted to coarse powder using mechanical grinder, then sieved through 20 # sieve. The coarse powdered plant material (2 Kg) was defatted with petroleum ether (3 L) using Soxhlet apparatus for 72 h. Defatted plant (1.6 Kg) material was extracted with ethyl acetate (3L) in Soxhlet apparatus for 72 h. The extract obtained was filtered and concentrated in rotatory evaporator (Perfit India, Pvt. Ltd.) below 60°C under reduced pressure. Concentrated extract (55 g) was stored in vacuum desiccator for several days to remove remaining traces of solvent until its use.

Column chromatography

Ethyl acetate extract of the seeds of *T. purpurea* 40 g was subjected to column chromatography in a cylindrical glass column (500 mm × 40 mm) using silica gel (80-120 #) as adsorbent. Column was packed with silica gel by wet packing method with petroleum ether. The gradient elutions were made by using different ratio of hexane: ethyl acetate (100: 0, 98: 2, 96: 4, 94: 6, 92: 8 and 90: 10) as mobile phase. All the fractions were collected in 100 mL conical flask and the fractions showing similar spots on thin layer chromatography (TLC) were mixed together after observing in long UV (365 nm), short UV (254 nm) and iodine chamber. The preliminary determination of spots was carried out by derivatization using either iodine vapours or 10% methanolic H_2SO_4. Hexane: ethyl acetate (98:2) fractions afford a compound TP-1 (56.0 mg) in the form of yellowish sticky solid. TP-3 (2.050 g) in the form of yellowish crystals was isolated from hexane: ethyl acetate (96:4) fractions. A compound TP-5 in the form of white color powder was obtained from hexane: ethyl acetate (90:10) fraction which after crystallization in ethanol gave needle shaped crystals (42.0 mg).

Quantitative estimation of TP-3 in EETP by HPLC

Quantitative estimation of compound TP-3 was performed by HPLC method as described by Gore and Satyamoorthy[29]. Methanol, acetic acid and water in the ratio of 85: 1.5: 13.5 used as mobile phase at a flow rate of 0.5 mL min^{-1} using Kromasil 100, C-18 column, ex Tracer (250 mm X 46 mm, particle size 5 microns) column. Instrument was equipped with UV-visible detector and absorbance of the compound was measured at 350 nm. Parsol MCX (2-Ethylhexyl-*p*-methoxy cinnamate) was used as the internal standard sunstance. The analysis was performed at Herbal Health Research consortium Pvt. Ltd, Village-Khayala Khurd, Amrisar, Panjab, India.

Anticancer activity of EETP and TP-3

Cell culture

SiHa cells were used for cytotoxicity studies. In 25 cm^2 tissue culture flasks, SiHa cells (1×10^6 cells) were cultured in and incubated in 5 mL DMEM (Dulbecco's Modified Eagle Medium), supplemented with 50 µL penicillin-streptomycin (1X), 10 µL plasmocin prophylactic (1X) and 500 µL heat inactivated fetal bovine serum (10% FBS) at 37°C in a humidified incubator with 5% CO_2 atmosphere. Cells were passaged at 75% confluence and growth medium was replaced every third day.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood obtained from healthy

donor. Peripheral blood (5 mL) from healthy volunteers was freshly collected in a heparinized falcon and diluted with PBS (phosphate-buffered saline) in 1:1 ratio. HiSep^{TM} LSM 1077 (1.66 mL) was carefully poured into two clean centrifuge tube (15 mL) over laid with 5 mL of diluted blood. Both the tubes were centrifuged for 30 min at 400 X g at room temperature (25°C). Supernatant containing plasma and platelet was discarded. Mononuclear cells carefully aspirated and transferred to a clean centrifuge tube and added with 5 mL of Isotonic PBS. Content of tubes were mixed and centrifuged at 200 X g for 5 min at room temperature. Washing step was repeated 3 times to remove HiSep LMS. During the second washing step cells were counted by using trypan blue exclusion assay. Cells (1×10^6 cells) were cultured at 37°C in a humidified incubator with 5% CO_2 atmosphere in 5 mLRPMI 1640 media, supplemented with 50 µL antibiotic-antimycotic solution (1X) and 500 µL heat inactivated FBS (10%).

Assessment of cell viability by MTT assay

The cytotoxicity of EETP and TP-3 on SiHa cells as well as PBMCs were determined by MTT assay. SiHa cells and PBMCs were seeded in 96 well microtiter plates (1×10^4 cells/well) and incubated overnight. EETP (25, 50, 75, 100, 125 and 150 µgmL^{-1}) and TP-3 (5, 10, 20, 30, 40 and 50 µg/mL) were added in cells separately in triplicates and incubated at 5% CO_2 atmosphere at 37°C for 24 h. Cells without test drugs were traeted as control. After incubation, each cells were added with 5 mgmL^{-1} of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltratrazolium bromide (MTT). After 4 h, 0.04 M HCl and isopropanol was added to the medium having MTT solution, and incubated at 37°C for 1 h in dark. Absorbance was measured at 570 nm using a 96 well microplate reader (Synergy^{TM} HT, Bio-Tek Instruments, Inc.). Mean absorbance (OD_{570}) values were plotted against different concentration of EETP and TP-3 used. Percent cell viability was calculated by using the formula:

Percentage cell viability =
[mean OD value of sample (treated)/ mean OD value of control (untreated)]* 100
% Cytotoxicity = 100 – % cell viability[30].

Statistical analysis

The differences between treatments and control were determined using one-way analysis of variance (ANOVA) using GraphPad Prism software (version 5.0), followed by a post-hoc test (Dunnett, $p<0.05$).

Results

Isolation and characterization of compounds

Compound TP-1 was isolated from the column chromatography of EETP. Column was eluted with the mixture of hexane and ethyl acetate in different ratio. Hexane: ethyl acetate (98: 2) fractions yielded TP-1 in the form of yellowish sticky solid. Physical properties and spectroscopic data of TP-1 are represented as-

R_f value= 0.6 (Hexane: Ethyl acetate, 9.5: 0.5); m.p. 28-30°C; IR bands (KBr, ν cm^{-1}): 3300 (O-H str), 2927 (C-H str), 2855 (C-H str), 1747 (C=O str), 1464, 1171, 1113; ^{1}H-NMR ($CDCl_3$, 400 MH_z):δ4.12 (2H, s, H-1), 1.60 (2H, s, H-2), 2.09 (2H, s, H-3), 2.29 (1H, t, *J*=7.2 Hz, H-5), 2.09 (1H, s, H-6), 1.30 (2H, s, H-8),1.25 (2H, s, H-9), 2.75 (1H, s, H-11), 2.76 (1H, s, H-12), 0.97 (1H, t, *J*=7.6 Hz, H-13), 0.89 (3H, s, H-14), 4.31, 4.30 (2H, H-15)**;** ^{13}C NMR ($CDCl_3$, 100 MH_z):δ C-1 (69.12), C-2 (32.15), C-3 (29.92), C-4 (130.45), C-5 (69.12), C-6 (62.32), C-7 (173.51), C-8 (27.42), C-9 (34.26), C-10 (34.42), C-11 (173.06), C-12 (22.79), 13 (14.49), C-14 (18.0), C-15 (127.34); HR-ESIMS: *m/z* (% rel. int.): 236 $[M]^+$ (5%), 195 $[M+H]^+$ (2.5%), 257 (2.6%), 212 (2.4%) 165 $[M]^+$ (1.5%).

Hexane: ethyl acetate (96: 4) fractions yielded TP-3, which was crystallized in ethanol as yellowish to white crystals. Physical and spectroscopic data of TP-3 are represented as-

R_f value= 0.6 (hexane : ethyl acetate, 9:1); m.p. 129-132 °C; UV Abs (MeOH, λ_{max}): 213, 348; IR bands (KBr, νcm^{-1}): 2919 (C-H str), 2853 (C-H str), 1739 (C=O str), 1597, 1477 (C=C str), 1358, 1223, 1058, 775, 699.7; ^{1}H-NMR ($CDCl_3$, 400 MH_z):δ7.58 (1H, d, *J*=2.4 Hz, H-5'), 7.99 (1H, d, *J*=1.6 Hz, H-6'), 6.99 (1H, d, *J*=0.8 Hz, H-7'), 7.61 (1H, d, *J*=2.4 Hz, H-8'), 7.98 (1H, d, *J*=1.2 Hz, H-2), 7.96 (1H, d, *J*=1.6 Hz, H-3),7.88 (1H, S, H-4), 7.96 (1H, d, *J*=1.6 Hz, H-5),7.98 (1H, S, H-6), 3.95 (2H, S, H-8), 4.64 (3H, S, OMe); ^{13}C NMR ($CDCl_3$, 100 MH_z): δ C-1' (119.58), C-2' (158.89), C-3' (122.37), C-4' (158.89), C-5' (105.28), C-6' (153.93), C-7' (107.25), C-8' (144.80), C-1 (135.5), C-2 (128.82), C-3 (127.30), C-4 (132.35), C-5 (127.71), C-6 (128.93), C-7 (186.31), C-8 (98.10), C-9 (194.66), Ome (60.26); HR-ESIMS: *m/z* (% rel. int.): 295.0955 [M+H] (100), and 317.0790 $[M+Na]^+$ (2.5), 217, 175.

Compound TP-5 was isolated from hexane: ethyl acetate (90: 10) fraction. It showed the similar R_f value (0.6; hexane: ethyl acetate 7:3) with *β*-sitosterol on TLC.

Quantitative estimation of TP-3 in EETP

The peak for TP-3 in EETP was found at the retention time 13.693 minutes in the HPLC chromatogram. The concentration of compound TP-3 in EETP was found to be 6.05% (Fig. 1 and Fig. 2).

Cytotoxicity of TP-3 and EETP

Isolated compound TP-3 showed cytotoxicity against SiHa cells with the IC_{50} 33.06 µgmL^{-1}. TP-3 showed cytotoxicity at all the concentrations but significant activity was observed at the concentration range from 20-50 µgmL^{-1} as compared to control. Maximum cytotoxicity (72.75%) was observed at the concentration of 50 µgmL^{-1} (Fig. 3). TP-3 showed non-significant cytotoxicity against PBMCs cells at all the concentrations except at 50 µgmL^{-1} (cytotoxicity 24.19%) (Fig. 4). EETP showed significant cytotoxic activity (IC_{50} value 113.63 µgmL^{-1}) against SiHa cells at the concentration range from 75-150 µgmL^{-1} as compared to control. Maximum cytotoxicity (75.34%) was observed at the concentration of 150 µgmL^{-1} (Fig. 5).

Discussion

Compound TP-1 was isolated from the column chromatography of EETP in the form of yellowish sticky solid. It gives positive color reaction with *p*-anisaldehyde-sulphuric acid reagent (violet colour), with the R_f value 0.6 (Hexane: EtOAc, 8:2) indicating the terpenoidal nature of the compound[1]. HNMR spectrum of the compound exhibited signals for a tertiary methyl [0.89 (3H, s, C-14)], an oxymethine [δ 4.12 (2H, s, C-1H)] and an exocyclic methylene [4.31, 4.30 (2H, C-15)][13]. C NMR spectrum and DEPT analysis of the compound showed the presence of 15 carbons. Its octahydro-1 *H*-indene carbon skeleton was suggested on the basis of its ^{1}H and ^{13}C NMR spectral data[31]. Molecular formula for the compound TP-1 was established as $C_{13}H_{24}O_2$ on the basis of *m/z* 236 $[M]^+$ with 5% relative intensity and other spectroscopic data. IR spectrum was observed having peaks at 2855 cm^{-1} and 2927 cm^{-1} for the presence of C-H streching and 1747 cm^{-1} for the carbonyl group (C=O) streching vibration in the compound. The peak at 3300 cm^{-1} in IR spectrum of the compound confirms the presence of -OH group. On the basis of all the spectral data analysis structure of TP-1 was identified as teclenone (Fig. 6 A).

Compound TP-3 was isolated from the column chromatography of EETP in the form of yellowish white crystals. It gives red color with ferric chloride indicates the phenolic nature of the compound. A band at 1739 cm-^{1} in the IR spectrum of the compound indicated the presence of ketone functional group. Presence of ketone group in the compound was also supported by two signals at 186.31 (C-7) and 194.66 (C-9) in ^{13}CNMR spectrum. UV- visible

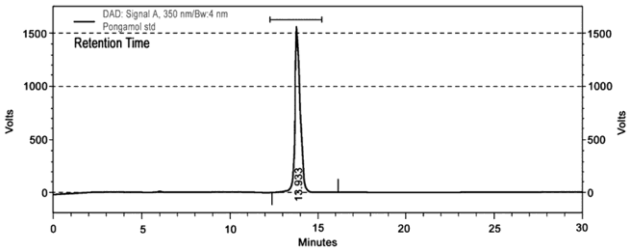

Fig. 1 — High performance liquid chromatography of pongamol standard

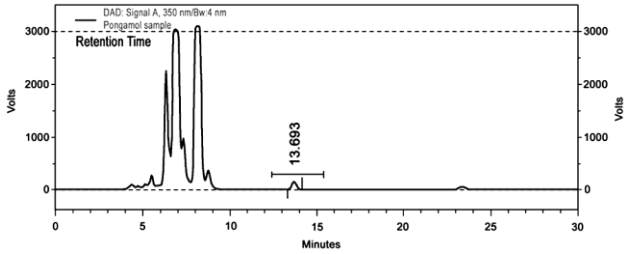

Fig. 2 — High performance liquid chromatography of EETP showing the presence of pongamol at retention time 13.693 min

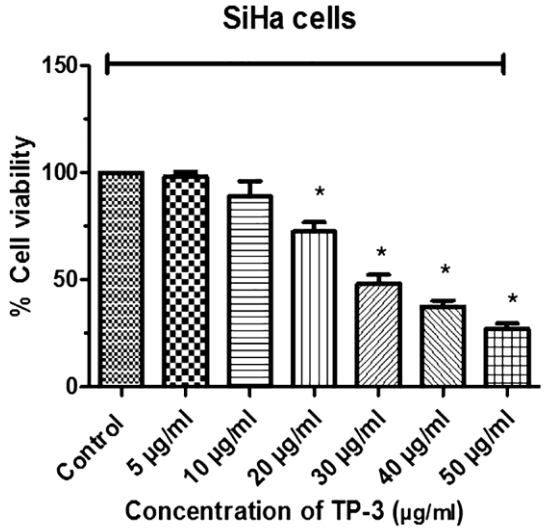

Fig. 3 — Effect of TP-3 on SiHa cells viability. SiHa cells were treated with different concentrations of pongamol (5, 10, 20, 30, 40 and 50 µgmL^{-1}) for 24 h. Cell viability % was analyzed using the MTT assay. Data represented as mean±SEM of three independent experiments done in triplicate. * $p<0.05$ vs. Control

spectrum of the compound showed λ_{max} value at 348 nm indicates the presence of conjugation system in the compound. In the ^{1}H NMR signals for two ortho-coupled aromatic protons [δ7.58 (1H, d, *J*=2.4 Hz, C-5'H), 7.99 (1H, d, *J*=1.6 Hz, C-6'H)], an unsubstituted ring [7.98 (1H, d, *J*=1.2 Hz, C-2H), 7.96 (1H, d, *J*=1.6 Hz, C-3H),7.88 (1H, S, C-4H), 7.96 (1H, d, *J*=1.6 Hz, C-5H),7.98 (1H, S, C-6H)] with 2 singlets and 3 doublets for 5 protons and a fused furan ring having doublet signals [6.99 (1H, d, *J*=0.8 Hz, C-7'H), 7.61 (1H, d, *J*=2.4 Hz, C-8'H)] were found in ^{1}HNMR spectrum of the compound. The structure of TP-3 (Fig. 6 B) drawn on the basis of interpretation of spectroscopic data.

Compound TP-5 gives positive color reaction with Liebermann Burchardt reagent and *p*-anisaldehyde sulphuric acid (pink color turns to blue on keeping) with the R_f value 0.6 (hexane: EtOAc, 8:2) and m.p. 136-137°C, indicating the steroidal nature of the compound. On the basis of direct comparison with authentic sample, structure of compound TP-5 was confirmed as *β*-sitosterol (Fig. 6 C).

The plant *T. purpurea* is used for the treatment of a number disorders including cancer[24-26]. Several phytochemical constituents including pongamol, isolonchocarpin, karanjin, lanceolatin B, kanjone, sitosterol, purpurin, pupuritenin, purpureamethide are reported from the seeds of the plant[5-6]. Since the plant *T. purpurea* is already reported to have anticancer activity and we have isolated pongamol in sufficient quantity (2.050 g, 6.05% of extract), an attempt has been made to find out the role of pongamol in the anticancer activity of the seeds of *T. purpurea*. *In-vitro* anticancer activity of EETP and pongamol was performed by using SiHa cells. Effect of pongamol on peripheral blood mononuclear cells (PBMCs) was also evaluated. Results of cytotoxicity study revealed that isolated compound showed

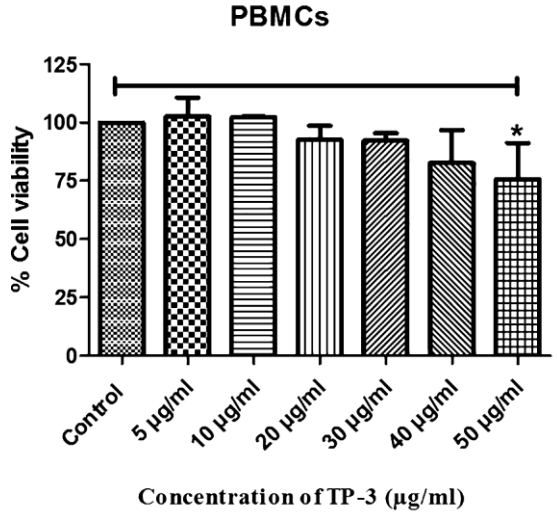

Fig. 4 — Effect of TP-3 on PBMCs viability. Cells were treated with different concentrations of TP-3 (5, 10, 20, 30, 40 and 50 µgmL^{-1}) for 24 h. Cell viability % was analyzed using the MTT assay. Data represented as mean±SEM of three independent experiments done in triplicate. * p<0.05 vs. Control

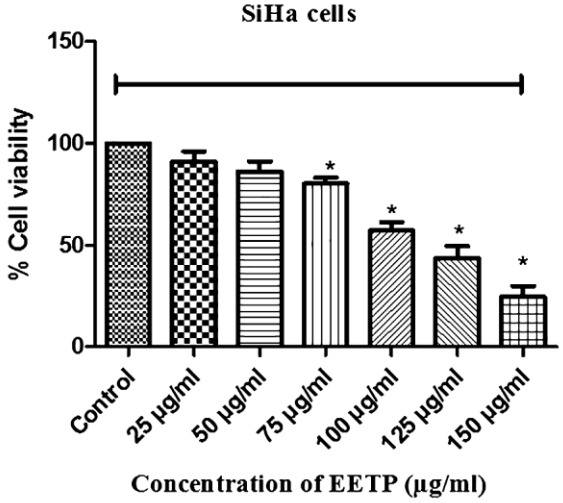

Fig. 5 — Effect of EETP on SiHa cells viability. SiHa cells were treated with different concentrations of EETP (25, 50, 75, 100, 150 and 150 µgmL^{-1}) for 24 h. Cell viability % was analyzed using the MTT assay. Data represented as mean±SEM of three independent experiments done in triplicate. * p<0.05 vs. Control

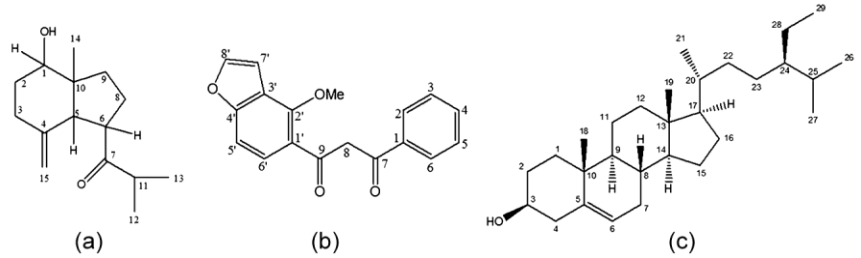

Fig. 6 — Structure of (a) teclenone (b) pongamol (c) *β*-sitosterol

cytotoxicity for SiHa cells (72.75% cytotoxicity at the concentration of 50 μgmL^{-1}) but found safe for PBMCs cells (24.19% cytotoxicity at the concentration of 50 μgmL^{-1} as compared to control). It means 75.81% PBMCs cells viability observed when treated with pongamol at 50 μgmL^{-1}.

Cytotoxicity of EETP was found better for SiHa cells as compared to TP-3 as per the percentage of compound in the extract. It means, along with the ponganmol other phytochemical constituents are also responsible for cytotoxic activity of EETP. Findings of the present study revealed that compound TP-3 (pongamol) could be a lead compound for cervical cancer and more active derivatives of pongamol may be synthesized in future with low toxicity against the normal cells.

Conclusion

In the present study, three compounds (teclenone, pongamol, *β*-sitosterol) were isolated from the seeds of *T. purpurea*. Teclenone was reported first time from the plant *T. purpurea* in best of our knowledge. Isolated compound pongamol showed cytotoxicity against cancer cell line (SiHa cells) and is responsible for the anti-cancer activity of *T. purpurea* along with the other phytochemical constituents.

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Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

Author's Contributions

SK, NC and NKS performed extraction and isolation of phytochemical constituents, analysis of spectroscopic data and elucidation of structure of isolated compounds. KP and GR performed anticancer activity using cell lines. DNS provide technical and administrative support for the study. All authors read and approved the manuscript.

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