In Vitro Antioxidant and Antiproliferative Efficacy of Abrus Precatorius Seed Extracts on Hep2c Cells and its Inhibitory Efficacy with Receptors of Cervical Carcinoma

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Abstract

Abrus precatorius is a widely distributed tropical medicinal plant with several therapeutic properties, however its seed extract has not been studied against cervical cancer (CaCx) till date. Herein, we have assessed the antioxidant and antiproliferative properties of *A. precatorius* seed extracts (ethyl acetate and 70% ethanol) prepared from different extraction methods (Soxhlet and maceration) against human cervix carcinoma cells (Hep2C). We observed a significantly higher total flavonoid content of APE (sox) i.e., 112.7 ± 0.127 mg Quercetin Equivalent/g of extract than others; total phenolic content of APA (mac) seed extract was higher i.e., 108.53 ± 0.089 mg Quercetin Equivalent/g of extract and total tannin content of APA (sox) was higher i.e., 98.98 ± 0.011 mg tannic acid equivalents/g of extract. In addition, tannic acid, rutin and piperine were identified in extracts by HPLC. Furthermore, APA (sox) exhibited the highest radical scavenging activity with an IC₅₀ value of $14.49 \pm 0.93 \mu\text{g/ml}$. APE (mac) showed the most significant antiproliferative activity with IC₅₀ value of $85.90 \pm 0.93 \mu\text{g/mL}$ against Hep2c cells. SOD and GST activity was observed as highest in the APA(Mac) extract whereas the catalase activity and GSH content was maximum in the APE(Mac) extract. Further, the MDA content was observed to be the least in APE(Mac) extracts. Docking results suggested maximum binding energy between tannic acid and Her2 receptor compared to doxorubicin (standard). This study provides evidence that *A. precatorius* seed extracts possess promising bioactive compounds with probable anticancer and antioxidant properties against CaCx which might be utilized as a possible herbal remedy aimed at restricting tumor growth.

Introduction

Cancer is one of the major causes of mortality in both men and women worldwide and its research has garnered attention from the scientific fraternity all across the globe. Considering the global burden of gynecological cancers, cervical cancer (CaCx) among them ranks as the fourth most common cancer with nearly 5,70,000 new reported cases and 3,11,000 deaths¹. Despite effective screening measures and treatment modalities, CaCx continues to hold the banner of the leading cause of cancer related mortality among women². Therefore, the development of novel therapeutic drugs with increased efficiency is warranted. Receptors like Glucocorticoids (GCR), Human epidermal growth factor receptor 2 (HER2) and Beta-hexosaminidase subunit beta (HEXB) are reported to be associated with progression of CaCx and have been established as promising therapeutic candidates as corroborated by *in-silico* analysis³⁻⁵. Out of the 1881 new chemical entities, that had been approved as drugs between 1981 and 2019, the purely synthetic compounds amount for only 24.6%, while a majority of the new drugs were derived from medicinal plants⁶. Herbal plants have made important contributions to the development of anticancer drugs. Numerous natural substances are recognized to be anti-oxidants, cancer preventive agents or even antitumor agents such as paclitaxel⁷.

Abrus precatorius L. (Fabaceae) is a plant that spans tropical and subtropical parts of the world. Lowly elevated, dry regions are conducive for its growth. Known for its medicinal value, its leaves, roots and seeds are often exploited for anti-helminthic, anti-diarrhoeal, neuroprotective, anti-depression, anti-fertility, anti-cataract, anti-arthritic, anti-allergic and anti-emetic purposes. *Abrus* derived lectins have been widely used in treating various cancers⁸. Evidence advocates that the seeds of this medicinal herb are effective in treating diabetes and chronic nephritis. Moreover, *A. precatorius* leaves possess a sweetness quotient equivalent to sucrose and are therefore used to sweeten foods in West tropical Africa⁹.

The main aim of the study was to prepare *A. precatorius* seed extracts using soxhlet and maceration methods to identify various phytochemical compounds and evaluate their abilities to function as antioxidant and *in-vitro*

assessment as anti-proliferative agent in human cervix carcinoma (Hep2c) cells.

Results

Molecular Identification

The amplified DNA was purified and sequenced using *rbcLa*-forward and *rbcLa*-reverse primers used for PCR. NCBI BLAST_N program was used to compare the nucleotide sequence available in Gen Bank. Sequence analysis confirmed the sample showed 98.8% identity having 81% query cover with previous reported sequences of *A. precatorius* (NC_047402.1).

Quantification of phytochemicals constituents

Total flavonoid content in seed extracts of *A. precatorius* were obtained from standard calibration curve $y = 0.010x$, $r^2 = 0.984$, where x is the concentration and y are the absorbance of quercetin ($\mu\text{g/ml}$) expressed in mg quercetin equivalents/ g of Extract as shown in Figure 1. APE (sox) extract demonstrated high flavonoid content i.e., 112.7 ± 0.127 mg quercetin equivalents/ g of extract, while APE (mac) extract showed 77.57 ± 0.002 mg quercetin equivalents/ g of extract. Moreover, APA (mac) extract exhibited 41.85 ± 0.017 mg quercetin equivalents/ g of extract while APA (sox) extract only showed 4.8 ± 0.014 017 mg quercetin equivalents/ g of extract (Table1) (Graph 1, Supplementary).

Furthermore, the phenolic content in seed extracts of *A. precatorius* as obtained from standard calibration curve $y = 0.015x$, $r^2 = 0.998$, where x is the concentration and y are the absorbance of is the absorbance of quercetin ($\mu\text{g/ml}$) expressed in mg quercetin equivalents/ g of extract as shown in Figure 1. Phenolic content of APA (mac) and APA (sox) seed extracts were found to be nearer to each other i.e., 108.53 ± 0.089 mg quercetin equivalents/ g of extract and 103.98 ± 0.036 mg quercetin equivalents/ g of extract, respectively. Whereas, APE (sox) and APE (mac) seed extract showed less amount of QE (quercetin equivalents) at 28.40 ± 0.157 mg / g of extract and 14.34 ± 0.045 mg / g of extract, respectively (Table1) (Graph 2, Supplementary).

Total tannins content in seed extracts of *A. precatorius* as obtained from standard calibration curve $y = 0.0143x$, $r = 0.9989$, where x is the concentration and y are the absorbance of is the absorbance of quercetin ($\mu\text{g/ml}$) expressed in mg tannic acid equivalents/ g of extract as shown in Figure 1. The results indicated that APA (sox) seed extract contained 98.98 ± 0.011 mg tannic acid equivalents / g of extract, while tannic acid equivalents value of APA (mac) seed extract was 89.35 ± 0.090 mg / g of extract Whereas, APE (sox) and APE (mac) seed extracts showed less amount of tannic acid equivalents at 26.35 ± 0.045 mg / g of extract and 17.07 ± 0.052 mg / g of extract, respectively (Graph 3, Supplementary).

Table 1: Quantitative estimation of phytochemicals of different seed extracts of *A. precatorius*.

Phytochemical Quantification	Ethyl acetate extract (Maceration Method)	Ethyl acetate extract (Soxhlet Method)	70% Ethanol extract (Soxhlet Method)	70% Ethanol extract (Maceration Method)
Total Flavonoid Content (mg Quercetin Equivalents /g of extract)	77.57± 0.002	112.7±0.127	41.85± 0.017	4.8±0.014
Total Phenolic Content (mg Quercetin Equivalents /g of extract)	14.34± 0.045	28.40±0.157	103.98± 0.036	108.53±0.089
Total Tannins Content (mg T.A Equivalents /g of extract)	17.07±0.052	26.35± 0.045	98.98± 0.011	89.35±0.090

Antioxidant Assay

The antioxidant potential of *A. precatorius* seed extracts were analyzed by DPPH free radical scavenging assay. Quercetin (standard) and the different seed extracts showed variable antioxidant properties. APA (sox) and APA (mac) seed extracts exhibited significantly ($p<0.00005$) high DPPH free radical scavenging in a dose-dependent manner with concentration (3-21 $\mu\text{g/ml}$) (Figure. 2 a) and (23-37 $\mu\text{g/ml}$) (Figure. 2 b). The IC₅₀ value of Quercetin was $14.49 \pm 0.93 \mu\text{g/mL}$. The higher IC₅₀ value indicates lower radical scavenging activity. The IC₅₀ value of APA (sox) and APA (mac) seed extracts were close to standard, i.e., $14.49 \pm 0.93 \mu\text{g/ml}$ and $23.8 \pm 0.68 \mu\text{g/ml}$, Figure. 2 a & b) respectively. Whereas, APE (sox) and APE (mac) seed extracts showing least DPPH free radical scavenging in a dose-dependent manner with concentration (150-350 $\mu\text{g/ml}$) (Figure. 2 c), and (150-650 $\mu\text{g/ml}$) (Figure. 2 d), with an IC₅₀ value $222.51 \pm 3.15 \mu\text{g/ml}$ and $482.76 \pm 6.96 \mu\text{g/ml}$, respectively (Figure. 2).

Antioxidant capacity was also quantified by FRAP assay. FRAP values were obtained against standard calibration curve of FeSO_4 at concentration between 100-1000 μM i.e., $y=0.001x$, $r^2=0.919$ where x is the concentration and y are the absorbance that indicates the e capacity to reduce ferric (III) iron to ferrous (II) iron. APA (mac) and APA (sox) have exhibited reducing capacity value $105060.2 \pm 0.086 \text{ mM Fe (II)/ g dry weight of seed extract}$ as and $95841 \pm 0.126 \text{ mM Fe (II)/ g dry weight of seed extract}$, respectively. APE (sox) and APE (mac) have revealed reducing capacity value $5849.39 \pm 0.014 \text{ mM Fe (II)/ g dry weight of seed extract}$ and $6216.59 \pm 0.005 \text{ mM Fe (II)/ g dry weight of seed extract}$, respectively (Graph 4, Supplementary).

HPLC analysis

Phytochemicals like polyphenolic flavonoids (rutin), tannins (tannic acid), and alkaloids (piperine) were determined in the seed extracts of *A. precatorius* prepared from different extraction method. The results show that the retention times of rutin, tannic acid and piperine were RT3.817, RT3.093 and RT14.847, respectively as shown in Figure 3. Rutin and tannic acid were identified in APA (sox), APA (mac), APE (sox), and APE (mac) seed extracts and their retention time were similar to the standards. Piperine was also identified but showed very less peaks in APA (mac), APE (sox) and APE (mac). No peak of piperine was identified in APA (sox) seed extract (Figure. 3). This indicates the presence of both the polyphenolic flavonoids and tannins in the seed extracts of *A. precatorius* prepared obtained from different extraction methods.

Cytotoxic activity of seed extracts against Hep2C Cell line

A. precatorius seed extracts were evaluated for its antiproliferative potential using MTT assay. Doxorubicin (standard) and the different seed extracts showed variable antiproliferative activity. IC50 values were also determined, lower IC50 values indicating higher the antiproliferative activity. As illustrated in Figure. 4 c, APE (mac) seed extract exhibited highly significant ($p<0.005$) cytotoxicity against Hep2C cells in a dose-dependent manner with concentration (50-200 $\mu\text{g/ml}$) with an IC50 value $85.90\pm0.93\mu\text{g/ml}$ as compared to the APE (sox) seed extract with an IC50 value $142.80\pm10.80\mu\text{g/ml}$. Moreover, APA (sox) and APA (mac) seed extracts showed less cytotoxicity against Hep2C cells as compared to APE seed extracts with an IC50 values $374.16\pm20.53\mu\text{g/ml}$ and $430\mu\text{g/ml}$, (Figure. 4 b) respectively. Comparatively, Doxorubicin, an anticancer drug used in this study as a positive control, demonstrated IC50 value $1.84\mu\text{g/ml}$ in a dose-dependent manner with concentration (5-15 $\mu\text{g/ml}$) (Figure. 4 a). Comparative statistical significance between the extracts (APA (sox), APA (mac), APE (sox), APE (mac) and doxorubicin (Figure. 4 d). Further, upon observation under the microscope, the Hep2c cells after exposure with standard (doxorubicin), APE and APA seed extracts displayed reduced size and abnormally shrunken, compared to untreated cells (Figure. 4 e-j).

Antioxidant Enzymes Activity Assay on Hep2C cells

The antioxidant activity of each extract was observed in the treated Hep2C cells with IC50 specific values to determine the intercellular free radical scavenging ability. From Table. 2., we can observe that the highest SOD enzymatic activity in APA (Mac) i.e., $4.6\pm0.08\text{ U/min/mg}$ of protein at an IC50 of $430.08\pm20.46\mu\text{g/ml}$ compared to the control cells i.e., $2.74\pm0.08\text{ U/min/mg}$ of protein and other extracts. Similarly, the catalase enzyme activity was observed to be the highest in the APE (Mac) extract with $28.37\pm0.32\mu\text{moles/min/mg}$ of protein at IC50 of $85.90\pm0.93\mu\text{g/ml}$ compared to the control i.e., $8.49\pm0.08\mu\text{moles/min/mg}$ of protein and other extracts. The non-enzyme content such as glutathione and lipid peroxidation were also quantified in the cells. The GST activity in the Hep2C cells after treating with the APA(Mac) extract was observed to be the highest ($42.16\pm0.55\mu\text{moles/min/mg}$ of protein, IC50: 430.08 ± 20.46) compared to the control ($28.5\pm0.31\mu\text{moles/min/mg}$ of protein) and other extracts. Similarly, we found that APE (Mac) treated cells with IC50: $85.90\pm0.93\mu\text{g/ml}$ (IC50) showed maximum glutathione content of $22,438\pm456.25\mu\text{g/mg}$ of protein compared to the control cells i.e., $18,397\pm57.42\mu\text{g/mg}$ of protein and other extracts. Hep2C cells showed a marked decrease in the MDA content ($0.041\pm0.0009\mu\text{g/mg}$ of protein) upon exposure to APE(Mac) with IC50 of $85.90\pm0.93\mu\text{g/ml}$ rather than the extracts and control cells i.e., $0.084\pm0.002\mu\text{g/mg}$ of protein (Figure. 5).

Table. 2. Enzymatic and Non-enzyme content in Hep2C cells upon treatment with different extracts

Extract	IC50 ($\mu\text{g/ml}$)	SOD (Unit/min/mg of protein)	Catalase ($\mu\text{moles/min/mg}$ of protein)	GST ($\mu\text{moles/min/mg}$ of protein)	GSH ($\mu\text{g/mg}$ of protein)	MDA ($\mu\text{g/mg}$ of protein)
Control	-	2.74 ± 0.08	8.49 ± 0.08	28.5 ± 0.31	$18,397.1\pm57.42$	0.084 ± 0.002
APA(Mac)	430.08 ± 20.46	4.6 ± 0.08	25.30 ± 0.08	42.16 ± 0.55	$20,615.8\pm93.18$	0.064 ± 0.001
APA(Sox)	374.16 ± 20.53	4.05 ± 0.05	23.15 ± 0.05	38.62 ± 0.79	$19,117.3\pm33.42$	0.079 ± 0.0006
APE(Mac)	85.90 ± 0.93	3.92 ± 0.28	28.37 ± 0.32	33.55 ± 0.55	$22,438.8\pm456.25$	0.041 ± 0.0009
APE(Sox)	142.80 ± 10.80	3.05 ± 0.06	12.57 ± 0.06	29.37 ± 0.38	$19,544.6\pm33.42$	0.072 ± 0.0008

Molecular Docking Analysis

Molecules characterized from HPLC were identified and docked to cervical cancer specific targets such as Human Epidermal Growth Factor Receptor 2 (HER2), Hexosaminidase Subunit Beta (HEXB) and Glucocorticoid receptor (GCR). Molecular docking was performed followed by MM-GBSA refinement using Doxorubicin as a control molecule. The results are given in Table 3. It was found from the study that tannic acid has scored the highest docking score of -112.53 Kcal/mol with HER2 (Figure.6 b). Piperine revealed a similar docking score of -42.11Kcal/mol and -42.15 Kcal/mol for Glucocorticoid receptor and HER2 respectively (Figure. 6 c). However, piperine resulted in a lower dock score of -29.74 Kcal/mol for HEXB. In the binding pocket of HER2, piperine forms one conventional and three aromatic hydrogen bonds. The Hydrogen bond formation occurs between the HN of Leu436 and O of 1,3-dioxolane ring of piperine (1.81 Å). Aromatic hydrogen bonds are formed with Gly28, Leu 436, and Arg434. Similarly, in the active pocket of GCR Piperine forms a single hydrogen bond with THR739. Rutin is a flavonoid that can be obtained by the glycosylation of quercetin. The docking score of rutin varied from -48.21Kcal/mol to -35.02 Kcal/mol. The rutin was found to have a higher binding affinity towards the HER2 receptor by forming 7 conventional hydrogen bonds within the active site. The interaction was mostly found due to the presence of OH groups in the structure. The active site residues involved in the formation of hydrogen bonds were Gln57, Gly28, Asp30, Arg34, Gly346, Arg351, Thr312, Tyr409 and Leu 436 (Figure. 6 a).

Table 3: Molecular Docking Score of HPLC characterized compounds against cervical cancer targets

Targets/Co Compounds	Piperine	Tannic Acid	Rutin	Doxorubicin
GCR	-42.11 Kcal/mol	NP	-37.11 Kcal/mol	NP
Her2	-42.15 Kcal/mol	-112.53 Kcal/mol	-48.21 Kcal/mol	-61.736 Kcal/mol
HEXB	-29.74 Kcal/mol	NP	-35.02 Kcal/mol	-53.905 Kcal/mol
*NP denotes production of no docking pose.				

The ADME and Druglikeness properties

The *in-silico* ADME and druglikeness prediction revealed that all the compounds have potential of being hERG blocker, while piperine can be hepatotoxic and may potentially have harmful reproductive effect. Also, it was observed among all the compounds tannic acid has the lowest drug score of 0.17 which is considered as very less for considering a compound as a lead molecule in clinical trials. Rutin and Tannic acid may also cause drug induced liver damage. Since results are based on a prediction model future in vivo studies are needed for validation (Table 4).

Table 4: ADME and Drug likeliness evaluation of HPLC characterized compounds.

	Molecules	Piperine	Tannic Acid	Rutin	Notes
Absorption	Papp (Caco-2 Permeability)	-4.661 cm/s	-6.525 cm/s	-6.606 cm/s	Optimal: higher than -5.15 cm/s
	HIA (Human Intestinal Absorption)	68.5%	19.9%	21%	≥30%: HIA+; <30%: HIA-
Distribution	PPB (Plasma Protein Binding)	89.021%	61.87%	76.65%	Significant with drugs that are highly protein-bound and have a low therapeutic index.
	VD (Volume Distribution)	0.579 L/Kg	-1.228 L/kg	-1.052 L/kg	Optimal: 0.04-20L/kg; Range: <0.07L/kg: Confined to blood, bound to plasma protein or highly hydrophilic; 0.07-0.7L/kg: Evenly distributed; >0.7L/kg: Bound to tissue components (e.g., protein, lipid), highly lipophilic
	BBB (Blood–Brain Barrier)	0.989	0.783	0.018	BB ratio ≥0.1: BBB+; BB ratio <0.1: BBB-These features tend to improve BBB permeation: H-bonds (total) < 8–10; MW < 400–500; No acids.
Metabolism	P450 CYP1A2 inhibitor	Positive	Negative	Negative	
	P450 CYP1A2 Substrate	Negative	Negative	Negative	
	P450 CYP3A4 inhibitor	Positive	Negative	Positive	
	P450 CYP3A4 substrate	Positive	Negative	Negative	
	P450 CYP2C9 inhibitor	Negative	Negative	Negative	
	P450 CYP2C9 substrate	Negative	Negative	Negative	
	P450 CYP2C19 inhibitor	Negative	Negative	Negative	
	P450 CYP2C19 substrate	Negative	Negative	Negative	
	P450 CYP2D6 inhibitor	Positive	Positive	Negative	
	P450 CYP2D6 substrate	Positive	Negative	Negative	
Excretion	T _{1/2} (Half Life Time)	1.483 h	2.443 h	2.138 h	Range: >8h: high; 3h< Cl < 8h: moderate; <3h: low
	CL (Clearance Rate)	1.965 mL/min/kg	-1.098	0.641 mL/min/kg	Range: >15 mL/min/kg: high; 5mL/min/kg< Cl < 15mL/min/kg: moderate; <5 mL/min/kg: low

Toxicity	hERG (hERG Blockers)	Positive	Positive	Positive	
	H-HT (Human Hepatotoxicity)	Positive	Negative	Negative	
	AMES (Ames Mutagenicity)	Negative	Negative	Positive	
	SkinSen (Skin sensitization)	Negative	Negative	Negative	
	LD50 (LD50 of acute toxicity)	1184.042 mg/kg	1643.452 mg/kg	419.47 mg/kg	High-toxicity: 1~50 mg/kg; Toxicity: 51~500 mg/kg; low-toxicity: 501~5000 mg/kg.
	DILI (Drug Induced Liver Injury)	Negative	Positive	Positive	
Osiris Property Explorer	Mutagenic	Negative	Negative	Negative	
	Tumorigenic	Negative	Negative	Negative	
	Irritant	Negative	Negative	Negative	
	Reproductive Effect	Positive	Negative	Negative	
	Drug Score	0.39	0.17	0.57	0-1

Discussion

Cervical cancer remains a burden for women of low- and middle-income countries (LMICs) such as India, South Africa, China and Brazil. Half a million new cases of cervical cancer and 311 365 deaths are reported annually due to lack of effective treatment ¹. Currently, the recommended therapeutic regimens include chemotherapy, radiation therapy, and surgery ¹⁰. However, they present several limitations including side effects or ineffectiveness. Therefore, it is important to search for new novel therapeutic agents or drug candidates that are naturally synthesized which will specifically act on the cancer cells without affecting the normal cells. Plant extracts and their bioactive compounds play a significant role in prevention of cancer and many more diseases. Phytochemicals have proved to be an excellent reservoir of polyphenols, tannins, flavonoids, alkaloids, terpenes, *etc.* ¹¹. Recently, more attention has been placed on tannins with the utilization of some herbs such as *Phyllanthus emblica*, *Sanguisorba officinalis*, as well as red wine with considerable tannins ¹². Plant-derived chemotherapeutic agents such as cisplatin, carboplatin, paclitaxel, ifosfamide, curcumin, camptothecin, taxol, and combretastatin have been used widely against cervical carcinoma ¹³. Keeping all the above points in mind present study was designed to evaluate ethyl acetate and 70% ethanol seed extracts of *A. precatorius* obtained by different extraction methods as a potential therapy against cervical carcinoma by evaluating its antioxidant capacity and *in-vitro* anti-proliferative activity as well as binding affinity of its polyphenolic flavonoids (rutin) and tannins (tannic acid) against receptors mediating signaling pathways of cervical carcinoma. Here, we reported that the content of major flavonoids of *A. precatorius* was significantly higher in APE (sox) seed extract (112.7±0.127 mg Quercetin Equivalent/g of extract) as compared to the other extracts. Similarly, the total phenolic content in *A. precatorius* was higher in APA (mac) seed extract (108.53±0.089 mg Quercetin Equivalent/g of extract) in comparison to the other extracts. Moreover, the total tannin

content of APA (sox) amounting to 98.98 ± 0.011 mg tannic acid equivalents/g of extract was higher than that of other extracts. As corroborated previously, less content of flavonoids, phenolics and tannins of *A. precatorius* have been reported. Another study by Jing *et al.*, reported that polarity of the solvents used for extraction plays an important role in the concentration of phenols and flavonoids¹⁴. HPLC can be used to identify, separate and quantify, phytochemicals¹⁵. The most important compounds identified in our study are polyphenolic flavonoids (rutin), tannins (tannic acid) and alkaloids (piperine). All these compounds contained in the seed extracts of *A. precatorius* may account for its antioxidant capacity. To evaluate antioxidant capacity, DPPH free radical scavenging was examined with 70% ethanolic and ethyl acetate seed extracts where APA (sox) seed extract exhibited excellent radical scavenging activity with an IC₅₀ value of $14.49 \pm 0.93 \mu\text{g/ml}$ as compare to other extracts. (Figure 2). Previously, Gul *et al.*, observed antioxidant potential of leaf extracts of *A. precatorius*, with an IC₅₀ value of $60.67 \pm 1.03 \mu\text{g/ml}$ ¹⁶. The radical scavenging activity can be explained by different composition of each extract as there are compounds (polyphenolic flavonoids and phenolics) that react quickly with DPPH to get reduced due to the formation of nonradical¹⁷. Therefore, as corroborated by previous studies, polyphenols and tannins could be beneficial for human health, owing to their antioxidant property^{18,19}. We have reported for the first time about the potential anticancer activity of *A. precatorius* seed extracts on Hep2c cells and its inhibitory efficacy with receptors of Human cervical carcinoma. In order to evaluate *A. precatorius* as a potential therapy for cervical cancer, all seed extracts prepared by using soxhlet & maceration method extracts were assayed against Hep2c Cells. All seed extracts exhibited anti-proliferative activity in a dose-dependent manner. However, our results revealed that the most promising extract was *A. precatorius* ethyl acetate seed extract (APE (mac)) which had an IC₅₀ value $85.90 \pm 0.93 \mu\text{g/mL}$ in the Hep2c cell line. Previous studies on vulpinic acid reported an IC₅₀ value $34.4 \mu\text{M}$ against cervical carcinoma cell line²⁰ which may be attributed to the type of solvents and methods of extractions used. The enzymatic activity and non-enzyme content were determined in Hep2C cells. Our current study revealed that SOD and GST activity was observed as highest in the APA(Mac) extract whereas the catalase activity and GSH content was maximum in the APE(Mac) extract. Further, the MDA content was observed to be the least in APE(Mac) extracts. Polyphenolic flavonoids, tannins and alkaloids possesses potent anticancer activities with multiple mechanisms, such as apoptosis, cell cycle arrest, and inhibition of invasion and metastases¹². However, to understand the exact mechanism of specificity against cervical cancer cells, further in-depth and extensive investigations are required. The antiproliferative effect of *A. precatorius* seed extracts may be attributed to the suppression of multiple oncogenic signaling pathways and tumor-promoting factors. In this analysis, we studied the following receptors like Glucocorticoids, Her2 and HEXB known to be associated with proliferation of cervical cancer³⁻⁵. Piperine, rutin and tannic acid exhibits a myriad of medicinal benefits such as antioxidant, anti-inflammatory, anticancer and neuro-protective effects²¹⁻²³. In this study, tannic acid (TA) has shown maximum binding energy against Her2 receptor rather than the standard drug doxorubicin. As far as the previous studies were concerned compounds like Tangeretin, wogonin, quercetin, and other flavonoids have shown less binding energy to the GC and Her2 receptor with respect to present studies^{24,25}.

The present study concludes that both the extraction methods (maceration & Soxhlet) were effective to obtain a maximum amount of biologically active phytochemicals. This information is supported by the results of HPLC of phytochemicals of the extracts and can be further use for the isolation of bioactive compounds from seeds of *A. precatorius* which might be utilized as a possible herbal remedy against restricting growth of cervical carcinoma in near future. This plant seed has promising compounds to be tested as potential anticancer and antioxidant drugs for treatment of cervical cancer. However, further investigations need to be done either to isolate the antioxidant compounds or to determine the *in vivo* biological activity of these extracts, cervical cancer models are needed for preclinical trials.

Methods

Chemicals

The analytical grade chemicals were purchased from Hi-Media and Merck, India. Standard drugs were purchased from Sigma-Aldrich, India. 2,4,6-tris(2-pyridyl)-s-triazine TPTZ and MTT reagent were procured from Merck, India. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and penicillin (5000 U/mL), streptomycin (2500 U/mL) from Gibco (USA).

Sample collection

Seeds of *A. precatorius* were collected from the Khari Baoli, Kucha Challan, Chandni Chowk, Delhi complies with relevant institutional, national, and international guidelines and legislation. Voucher specimens were deposited at Raw Material Herbarium and Museum, Delhi (RHMD), India and were authenticated by Dr. Sunita Garg, (Emeritus Scientist, CSIR-NISCAIR), with Ref No: NISCAIR/RHMD/Consult/2020/3697-98-2.

Genomic DNA isolation and molecular identification

Genomic DNA was isolated from seeds of *A. precatorius* using Cetyltrimethylammonium bromide (CTAB) method ²⁶. The genomic DNA was amplified using *rbcLa*-forward primer (ATGTCACCACAAACAGAGACTAAAGC) and *rbcLa*-reverse primer (GTAAAATCAAGTCCACCRCG). PCR amplification was carried out in Veriti model of Applied Biosystem Thermo cycler ²⁷.

Cell culture

Human cervix carcinoma Hep2C cell line was obtained from National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂.

Preparation of Extracts

Seeds were washed with distilled water to remove dirt and soil particles, followed by drying and grinding to form powder and used throughout the study.

1. Soxhlet Extraction Method

Alcoholic extracts were prepared by soxhlet method using ethyl acetate and 70% ethanol as solvent. 10g of seed powder was placed inside a thimble made from thick filter paper and loaded into the soxhlet extractor. The Soxhlet extractor was placed onto the flask containing the solvent (500 mL) equipped with a condenser. The extractor was then allowed to heat to reflux for 16 h at 70 °C. Extracts were filtered twice through a Whatman No.1 paper filter and concentrated to the dry mass with the aid of rotary evaporator ²⁸.

2. Maceration Extraction Method:

10g of seed powder was dissolved in 100ml of solvent (Ethyl acetate and 70% Ethanol) and then kept in the dark for 7 days at room temperature. The conical flasks of the extract were covered with cotton plugs to avoid the evaporation. After 7 days of incubation, they were filtered with muslin cloth followed by Whatman No.1 filter paper and concentrated to the dry mass with the aid of rotary evaporator ²⁹.

The dried extracts were dissolved in absolute dimethyl sulfoxide (DMSO) as 50 mg/mL and diluted with phosphate-buffered saline (PBS, pH 7.4) to give final concentrations.

Quantification of Phytochemical Constituents

Total flavonoid content was measured by Aluminium Chloride Spectrophotometric method. Absorbance was measured against the prepared blank at 510 nm and results were represented as quercetin equivalents (mg QE)/g of extract. Similarly, total phenolic content and total tannins content was quantified by Folin-Ciocalteu method. Absorbance of mixture was measured at 725 nm. Final results were represented as quercetin equivalents (mg QE)/g of extract and tannic Acid equivalents (mg QE)/g of extract, respectively ^{28,30,31}. All the concentrations were calculated using a standard calibration plot.

Antioxidant Assay

Antioxidant potential in the seed extracts was determined by electron transfer assay i.e. (2,2-Diphenyl-1-picrylhydrazyl) Radical scavenging assay (DPPH) and Ferric reducing antioxidant power (FRAP) assay. DPPH free radical scavenging assay was performed to measure the hydrogen donating or radical scavenging ability. Briefly, 0.04mM DPPH radical solution was prepared in methanol and then 900 μ L of this solution was mixed with 100 μ L of extract solution containing different concentrations of seed extracts. The absorbance was measured at 517 nm after 30 min of incubation. Methanol (95%), DPPH solution and were used as blank, and control respectively. Quercetin was used as the standard. 50% inhibitory concentrations (IC₅₀ values) of the extracts were calculated from graph as concentration versus percentage inhibition. Radical scavenging activity was expressed as percentage of inhibition. Measurements were taken in triplicate. The IC₅₀ of the extract and standards were determined graphically ³².

The percentage of inhibition was calculated by using the formula:

$$\text{Percentage of inhibition} = \left[\frac{(\text{absorbance of control} - \text{absorbance of reaction mixture})}{\text{absorbance of control}} \right] \times 100$$

Further for the FRAP assay, FRAP reagent solution was prepared with 300 mM sodium acetate buffer (pH 3.6), 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl and 20 mM FeCl₃·6H₂O (10:1:1 v/v/v). The absorbance was measured at 593 nm after a 30 min incubation at room temperature against 50% ethanol as blank. A calibration curve was prepared using FeSO₄·7H₂O. FRAP activity was expressed as mM Fe (II)/ g dry weight of extract ^{28,33,34}.

High Pressure Liquid Chromatography

The presence of phenolic compounds and alkaloids in the prepared extracts was screened against standards rutin, tannic acid and piperine by HPLC. The analysis was performed using a C-18 reversed phase column (Phenomenex, Gemini 5 μ , 250 mm length $\times$ 4.6 mm internal diameter). The mobile phase consisted of methanol: 0.1% orthophosphoric acid (77:23) for quercetin, methanol: water (50:50) for tannic acid and 1% acetic Acid: acetonitrile (52:48) for piperine were chosen for the separation at a constant flow rate of 1 mL/min. The column temperature was set to 38°C and the injection volume was 20 μ L. The wavelengths were set to 370 nm for the detection of rutin, 280 nm for tannic acid, and 343nm for piperine. Retention time was observed by using HPLC ^{28,35}.

Antiproliferative activity

Cytotoxic activity of *A. precatorius* seed extracts on Hep2C cells were determined by using a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) assay. Briefly, Hep2c cells were seeded ($\sim 1 \times 10^4$ cells/well) onto flat-bottomed 96-well culture plates. Further, different concentrations of seed extracts and doxorubicin (standard) were

added to each well. After 48 h of incubation 10 µl of MTT reagent (5 mg/ml) were added and mixtures were re-incubated for 4 h. The resulting formazan was solubilized with DMSO (100 µl). Finally, the absorbance of formazan was measured at 570 nm using an automated microplate reader (Bio-Rad, Illinois, USA). Experiments were carried out in triplicates. The cytotoxicity was recorded as concentration causing 50% growth inhibition (IC₅₀) for cell line³⁶.

The percent inhibition was calculated by using the following formula:

$$\% \text{Inhibition} = 100 - (\text{mean OD of test compound} - \text{mean OD of negative control}) / (\text{mean OD of positive control} - \text{mean OD of negative control}) \times 100$$

Antioxidant Enzyme Activity Assay on Hep2C cells

To estimate the effect of *A. precatorius* seed extracts on Hep2C cells, the enzymatic activity [superoxide dismutase (SOD), catalase (CAT), Glutathione-S-Transferase (GST)] and non-enzyme content [Glutathione content (GSH) and lipid peroxidation (Malondialdehyde (MDA) content)] were evaluated. A total of 1.51×10^5 cells/well were seeded in 24 well plates and incubated for 24 h in a CO₂ incubator at 37 °C followed by a 48 h treatment with the obtained IC₅₀ specific values of each of these extracts. The cells were harvested by washing with PBS followed by trypsinization. Further, the samples were centrifuged at 10,000 rpm for 20 min at 4 °C to obtain the cell lysate which was further used for estimation of enzyme and non-enzyme content in accordance with standardized procedures³⁷.

Molecular Docking and MM-GBSA refinement

The molecular docking was performed on cervical cancer targets using molecules found in HPLC. The X-ray crystallographic structures of targets were obtained from Protein Data Bank (<https://www.rcsb.org/search>) using PDB ID:3LMY (HEXB), 5MY6(4LSJ), and HER2 (5MY6). Molecular docking was performed using Schrödinger Maestro Suite 2020-3 (Schrödinger, LLC, New-York, NY)^{38,39}. All the structures were optimized prior to docking, any structural inconsistencies were rectified using Schrodinger's in-built protein preparation wizard module. HEX-B and GCR both have co-crystallized ligands which were chosen to act as the center of the receptor grid. In case of HER2 active site prediction was made using SiteMap module, active sites with a score <0.9 were rejected. The internal grid size x*y*z was fixed 15*15*15 Å. Further, MM-GBSA refinements were carried out on the docked poses of each isoform with a flexible residue distance of 5.0 Å.

ADMET Analysis and Drug Score

The computational ADME/TOX analysis for identified compounds for 23 molecular descriptors were computed using ADMETLAB (<http://admet.scbdd.com/>)⁴⁰. Additional Descriptors like mutagenic and drug score were computed using Osiris Property Explorer⁴¹.

Statistical analysis

The statistical data were represented as mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA). A probability value of ≤ 0.05 was considered as statistically significant. All analyses were performed using IBM-SPSS statistical software (version 13.0).

Abbreviations

APE (sox): ethyl acetate extract soxhlet; **APA (mac)**: 70% ethanol extract maceration; **APA (sox)**: 70% ethanol extract soxhlet; **APE (mac)**: ethyl acetate extract maceration; **MTT assay**: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-

tetrazolium bromide; **SOD**: Superoxide dismutase; **CAT**: Catalase; **GST**: Glutathione s transferase; **GSH**: Glutathione; **MDA**: Malondialdehyde.

Declarations

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Author Contribution

Kumud Bala: Conceptualization, Methodology, Supervision. **Amritpal Kaur**: Experimental, Analysis, Writing- Original draft preparation, Methodology, Investigation. **Yash Sharma**: Visualization, Investigation. **Kamran Waidha**: Software, Statistical analysis, Bioinformatics, Validation. **Madhumita P Ghosh**: Writing- Reviewing, Editing, Supervision. **Anoop Kumar**: Statistical analysis, Validation

Consent for Publication

Not Applicable

Data availability

All data are available in the manuscript

Data repositories

Not applicable

Conflict of Interest

The authors declare that they have no conflicting or competing interests.

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Figures

Figure 1

Comparative quantitative analysis of phytochemical constituents presents in 70% ethanol and ethyl acetate seed extracts prepared from soxhlet and maceration extraction methods.

Figure 3

HPLC Chromatograms of (a.) standards: tannic acid, rutin and piperine, (b.) 70% ethanolic and ethyl acetate seed extracts of *A. precatorius* prepared from soxhlet extraction method showing rutin, tannic acid and piperine and (c.) 70% ethanolic and ethyl acetate seed extracts of *A. precatorius* prepared from maceration extraction method showing rutin, tannic acid and piperine.

Figure 4

Antiproliferative activity of doxorubicin (standard) and *A. precatorius* seed extracts against Hep2C Cells using MTT assay after 48hr of treatment. (a.) doxorubicin, (b.) APA (sox) and APA (mac), (c.) APE (mac) and APE (sox). (d.) Comparative statistical significance between the extracts (APA (sox), APA (mac), APE (sox), APE (mac)) and doxorubicin. Pictorial representation of Hep2C Cells under inverted light microscope using 10X objective lens (magnification 100X) after 48h exposure of doxorubicin and *A. precatorius* seed extracts. From 'e.' to 'j.' where (e.)

control Hep2C Cells (Untreated), (f.) Hep2C cells treated with doxorubicin, (g-h.) Hep2C cells treated with APE (mac); APE (sox) and, (i-j.) Hep2C cell treated with APA (sox); APA (mac). *p<0.05, **p<0.005, ***p<0.0005, ****p<0.00005

Figure 5

Antioxidant activity *A. precatorius* seed extracts in Hep2C cells on the enzyme (SOD, CAT & GST) and non-enzyme content (GSH and MDA). The data is representative of 3 consecutive experiments. *P<0.0.5, **P<0.01,***P<0.001

Figure 6

Molecular docking of (a.) Rutin, (b.) Tannic acid, (c.) Piperine and their interaction with amino acids of GCR receptor, HER2 receptor and HEXB receptor of cervical carcinoma.

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