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Influence of Solvent Polarity and Extraction Methodology on Recovery of Furanoflavanoids from *Pongamia pinnata* and Therapeutic Efficacy Against Skin Cancer

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Abstract

Current global requirements necessitate the successful achievement of maximum possible process efficiency, minimum possible usage of organic solvents and along with sustainability, and cost-effectiveness. These criteria become even more stringent if the raw material is based on plant-derived bioactive compounds as they are often present only in trace amounts. For the extraction process the above factors are majorly determined by the selection of solvents with respect to get the highest possible recovery and the selection of suitable extraction methodology. Furano-flavonoids such as pongamol and karanjin from the seeds of Pongamia pinnata (P. pinnata) shows considerable activities as skin care ingredients. However, the content of these furano-flavonoids is significantly less in the seeds, calls for the effective process development to achieve highest process recovery along with reduction of solvent volumes and optimized extraction methodologies for highest possible process efficiency and minimum possible process cost. The current study focuses to develop a suitable extraction solvent system among the - non-polar, mid-polar, and polar – solvents. The study also aims to optimize the extraction techniques among maceration, hot extraction, and Soxhlet extraction on the basis of extraction yield, furano-flavonoid recovery, and radical scavenging activity analysis by the DPPH assay. Furthermore, this study evaluates the therapeutic relevance of the optimized extract was evaluated through cytotoxicity analysis by MTT assay against skin cancer cells. From the key findings of this study, it was confirmed that solvent polarity and extraction techniques have a critical role in the development of sustainable and efficient process development. Extraction with hexane along with Soxhlet extraction methodology showed the highest efficiency in terms of yield, process efficiency and anti-skin cancer activity.

Keywords

Anti Skin cancer, Antioxidant, Karanjin, Pongamia pinnata, Pongamol, Sustainable process development.

INTRODUCTION

The largest organ of the human body, skin, is under constant strain from various environmental conditions, in which solar radiation accounts for the most significant one. In order to get mitigation from these stress factor, incorporation of efficient skin-care ingredients, specifically molecules with UV-absorption characteristics, are essential. There are few natural molecules which have UV absorbing capability such as lignin, quercetin etc., reported for their therapeutic usages for skin care activity. Sustainability, feasibility and viability of these ingredients are yet to establish in a commercial way, and scientists are in quest for unique natural molecules for this specific application area. *P. pinnata* seed oil was used for its skin care functionality from ancient times, and it is well documented in Ayurvedic and Siddha texts.

Active components of *P. pinnata* seed oil - furano-flavonoids: karanjin and pongamol - are the major active components behind these activities [1,2]. It was also observed that both these molecules showed prominent UV absorption characteristics. Karanjin has its maximum absorption at 300 nm and as pongamol showed the wavelength maximum at 350 nm [3]. Both these compounds together could cover a broad UV range with this absorption maximum as majority of the UV radiation is in the range of 280 to 380 nm range. The ability to absorb with this wide range of spectrum directly pointed out to the ability of these molecules with regards to its suitability as a unique sunscreen ingredient for broad spectrum photo-stabilization [4-6]. Usually, the commercial production of *P. pinnata* seed oil has been carried out by mechanical expelling process and region to region there were cold or hot expelling practices [7,8]. The conventional expelling process is not efficient to pull out the complete extractives and active compounds. By optimization with process intensification, there are opportunities for bringing higher recovery of overall extractives along with active components.

Affinity between active compounds and the extraction solvents are the major factors that determine the process efficiency. This affinity between those molecules majorly depends on dielectric compatibilities and intrinsic polarities [9,10]. Differences in the solvent polarities made variations in partition coefficients, diffusion rates, and also the disruption of plant cellular structures and played the major

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role in overall recovery of target phytochemicals. Extraction methodology is the base of the point that a particular preparation process is viable or not and without this viability no process will ever come to a sustainable level. Process techniques are directly connected with parameters like extraction kinetics, mass-transfer efficiency, solvent utilization, and operational simplicity etc. [11]. Extraction methodologies define the scalability levels of a process, product cost, and overall sustainability of the product.

Based on the above information, the current study aims to investigate the effects of solvent polarities on extraction yield, recovery of major furano-flavonoids - pongamol and karanjin from *P. pinnata* seeds, and the antioxidant activities of these extracts. The study also focuses on the anti–skin cancer activity of the seed extract obtained through the optimized extraction methodology with selected solvent.

MATERIALS AND METHODS

Plant Materials and Chemicals

P. pinnata seeds were acquired from Sangli, Maharashtra, India. Reference standards of pongamol and karanjin were purchased from Toronto Research Chemicals Inc., Canada. Hexane, acetone, ethanol, and HPLC-grade water was analytical-grade and were purchased from Merck (India). Methanol, and formic acid, both LR and HPLC grade, were purchased from Merk.

Effects of Solvent Polarity

Dried seeds obtained were flaked using an industrial lab-scale flaker for getting a uniform shape and size for all the input materials of all the treatments.

Selected four solvent systems - hexane, acetone, ethanol, and water – coming under nonpolar to polar solvents, for finding out the optimum solvent polarity which provide the highest yield and recovery. Different extraction process was done by taking accurately weighed 100 g of flaked *P. pinnata* seeds. Flaked seeds were charged into four separate flat bottom glass flasks having ground joints. Add 1:4 (that is 400 mL) of different solvent system (hexane, acetone, ethanol, and water) and close the flask using cotton to reduce the evaporation. Extraction was done by keeping at 25–30° C for 30 min. The miscella was then separated by decantation and filtered through Whatman grade 1 filter paper. The washing step was repeated three more times, and the solvent fractions from each cycle were pooled to obtain the combined extract for each solvent system. The solvent was evaporated separately using a rotavapor (Buchi, Switzerland) and the process started under atmospheric pressure and ended under a vacuum of 700 mmHg. Complete removal of solvent was confirmed by holding the material in a vacuum for 30 min. The process yields were determined by comparing the amount of extract obtained to the initial weight of the flaked seeds.

Effects on Extraction Methodology

Maceration extraction trials did as per the method mentioned in the above session. Hot extraction of the seeds done by charging 100gms of flaked seed material along with 400 ml hexane in a round bottom flask connected with a reflux condenser. Place this system in a water batch and maintain the water batch temperature at 60° C keep the system at that temperature for 30 min. Decant off the wash and continue to take three more washes in similar manner. The solvent was evaporated using a Buchi rotary evaporator. The process was initiated under atmospheric pressure and gradually reduced to a final vacuum of 700 mmHg. Complete solvent removal was ensured by maintaining the material under vacuum for an additional 30 minutes. Extraction yield was calculated by comparing the mass of the recovered extract with the initial weight of the flaked seeds.

Soxhlet extraction was done as mentioned by Bala et al., (2011) by fixing the solvent system to hexane and time of extraction to 4 Hrs. [12]. The solvent was removed using a Buchi rotary evaporator. Evaporation began under atmospheric pressure and gradually adjusted to a final vacuum of 700 mmHg. Complete solvent removal was confirmed by holding the material under vacuum for an additional 30 minutes. The extraction yield was determined by comparing the mass of the recovered extract with the initial weight of the flaked seeds.

Analysis of Furanoflavnoids - Pongamol and Karanjin - Content

The concentration of pongamol in the seed extracts was quantified using the HPLC method as described by Gore et al. (2000) with slight modifications [13]. An Acquity UPLC H-Class Plus system with an Acquity photodiode array (PDA) detector from Waters Corporation, USA, was used for the analysis. A C18 column (5 µm) with dimensions 4.6×250 mm was employed for chromatographic separation, and the mobile phase consisted of methanol/water/acetic acid (85/13.5/1.5) with an isocratic flow rate of 0.5 mL/min. The absorbance wavelength was set at 350 nm and 300 nm. The peak areas at 300 nm were used to quantify the karanjin content in the samples and peak areas at 350 nm were used to quantify the pongamol content. The karanjin standard chromatogram showed a retention time of 10.2 min and pongamol standard chromatogram showed a retention time of 13.4 min.

DPPH Radical Scavenging Assay

Antioxidant activity of the samples was measured by evaluating the effectiveness of quenching a stable free radical, 2,2-diphenylpicrylhydrazyl (DPPH) [14]. A standardized solution of DPPH was prepared by dissolving 0.02 g of DPPH in 100 mL of methanol, then 10 mL of this solution was pipetted out and made up to 100 mL with methanol. The test solution was prepared by dissolving the sample in adequate DMSO. Different concentrations of the sample and the standard were transferred to a 96-well

Journal of Pharmaceutical Research and Innovation (JPRI)
Volume 6, Issue 2, July 2026
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microplate (100 µL/well), and 100 µL of freshly prepared ethanolic ferric chloride solution was added to each well. The plate was kept for 30 minutes in the dark at room temperature, and the absorbance was measured at 517 nm using a SpectraMax i3X multi-mode microplate reader. The percentage inhibition was calculated for each concentration of the sample, and a graph was plotted using the percentage inhibition on the y-axis and the sample concentration on the x-axis. The IC_{50} value was calculated by using the equation of the line obtained by plotting the graph. All tests were performed in triplicate.

Anti Skin Cancer Study by MTT Assay

We obtained human skin cancer cells (A375) from the National Centre for Cell Sciences, Pune, India. These cells were then cultured in DMEM media, which was further fortified with 10% fetal bovine serum, 100 µg/mL penicillin, and 100 µg/ml streptomycin. The cells were then incubated at 37° C in an atmosphere containing 5% CO_2 and 95% humidity. The cells were sub cultured once they attained an 80-90% confluence and media were refreshed after every third day.

Cytotoxicity of the test materials was performed by MTT (3-(4,5-Dimethylthiazol-2-yl)- 2,5 Diphenyltetrazolium Bromide) based metabolic assay. Approximately 1x10s cells/ml- were seeded in a 24-we11 plate with complete growth medium and allowed to attach and grow. At 80% confluence, the medium was replaced with fresh medium containing different concentrations of test materials (0-100 µg/mL) and incubated for 48 hrs. At the end of the incubation period, the medium was replaced with fresh medium. Then 40 µL of 5mg/mL MTT was added to each well and incubated for 4 hrs. The formazan crystals formed were dissolved in dimethyl sulfoxide and the absorbance was measured at 570 nm using microplate reader (BioTek, USA). The percentage viability was calculated using the formula.

$$\%\,Viability = (Absorbance\ of\ Sample \div Asborbance\ of\ Control) \times 100 \quad (1)$$

RESULTS AND DISCUSSION

Effect of Extraction Solvents on Oil Yield

As shown in "Figure. 1", different percentage yields were observed with various solvent systems used for the extraction *P. pinnata* seeds. Extraction with hexane showed an average yield of 21.90%, while acetone and ethanol exhibited an average yield of 19.0 and 10.9%, respectively, and water showed an average yield at 12.4%. When compared with the lowest to highest it was observed a 101% hike in the extraction yield. This showed the significance of polarity of solvent system – hexane - in the extraction of *P. pinnata* seeds.

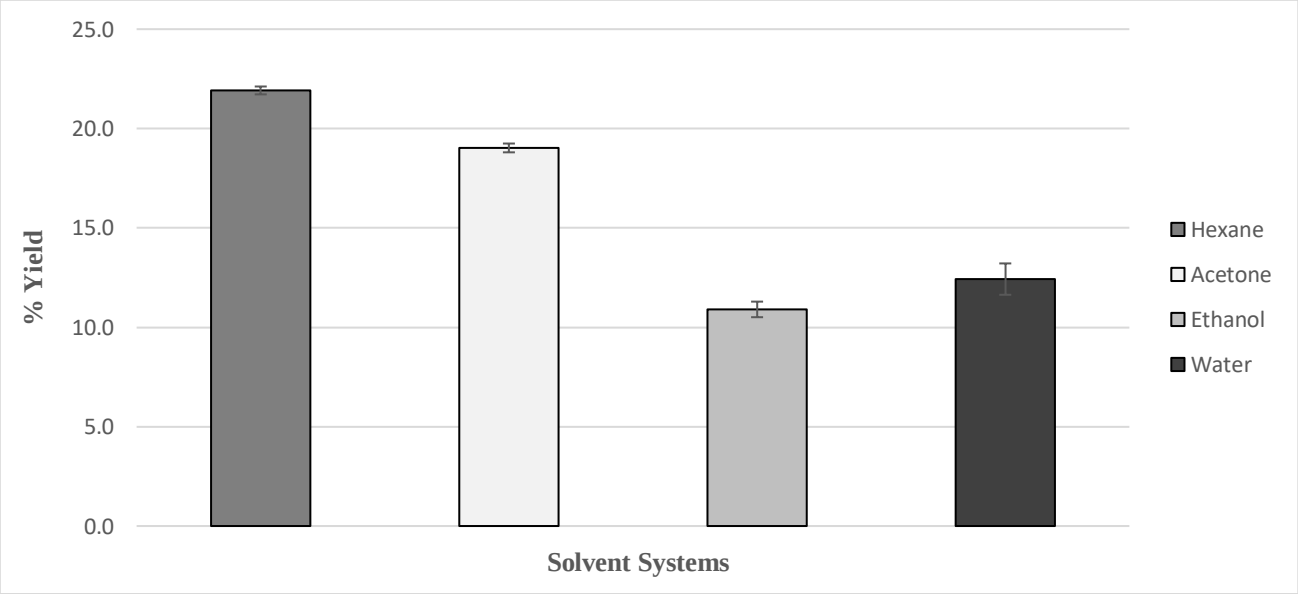

Figure 1. Effect of different extraction solvents on extraction yields from *P. pinnata* seeds (Data are presented as mean ± standard deviation of the mean (n = 4))

The detailed experimental data were compiled in Table 1 to provide a clear and systematic summary of the extraction yields obtained using the different solvent systems. Relative standard deviation (RSD) for extraction with the hexane solvent system showed 0.9% and this confirmed the high confidence level of the process with the backup of strong reproducibility and statistical reliability. The set data of extraction trials with acetone as solvent also showed an RSD less than 2% confirmed the reproducibility of the process. At the same time, extraction with ethanol and water showed higher than 2% RSD showed that processes have slightly less reproducibility when compared with hexane or acetone extraction systems. This higher value for the RSD may be due to the variations in partition coefficients with non-polar solute from the seeds and the polar solvent molecules. Penetration patterns of these highly polar solvent molecules

Journal of Pharmaceutical Research and Innovation (JPRI)
Volume 6, Issue 2, July 2026
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to the seed cavities also might be different than with the hexane or acetone.

Table 1. Yield of extract with different extraction solvents

Solvent	*Yield (%)*				*Average*	*SD*	*RSD*
	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**			
Hexane	22.1	21.8	21.7	22.07	21.9	0.2	0.90
Acetone	19.3	18.8	18.9	19.1	19.0	0.2	1.17
Ethanol	10.4	11.1	10.8	11.3	10.9	0.4	3.59
Water	12.5	13.1	11.3	12.8	12.4	0.8	6.35

Effect of Extraction Solvents on Furano Flavonoids Contents

Content of pongamol in the *P. pinnata* seed extract obtained through the extraction with different solvent treatments shown in "Figure. 2". Hexane extraction yielded highest pongamol content with 0.32%, while acetone and ethanol produced extracts with 0.27% and 0.22%, respectively, and water gave the least value of 0.01% pongamol content. The pongamol content increased with the reduction of polarity of solvents used for the extraction.

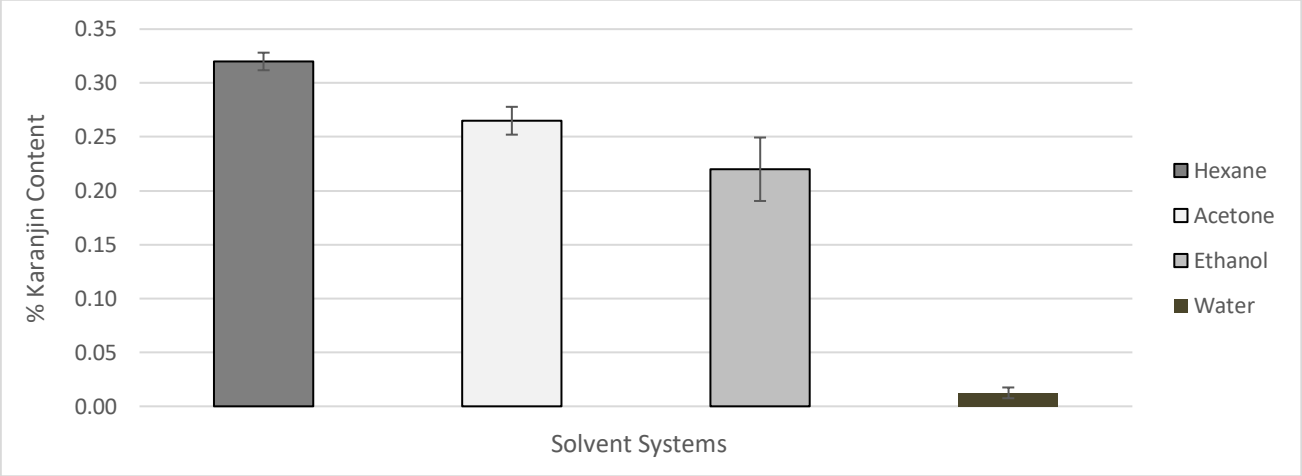
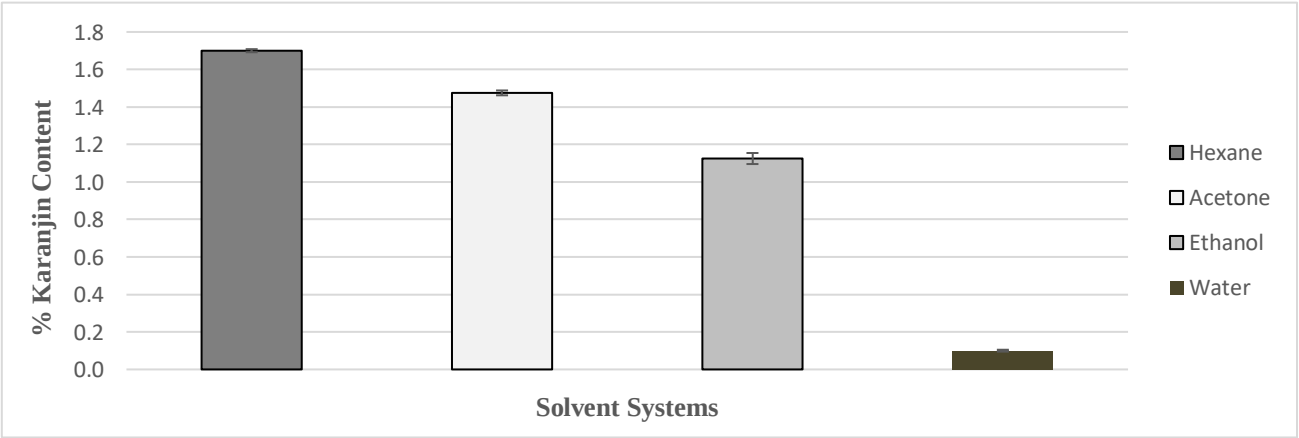


Figure 2. Effect of different extraction solvents on pongamol contents in extracts (Data are presented as mean ± standard deviation of the mean (n = 4))

The concentration of karanjin present in the *P. pinnata* seed extracts obtained using different solvent systems is presented in "Figure. 3". Among the solvents evaluated, hexane extraction yielded the highest karanjin content (1.7%), followed by acetone (1.5%) and ethanol (1.1%), whereas water extraction resulted in only 0.1%, representing the lowest recovery of the compound.

From both Figure 1 and Figure 2 it was confirmed that the solvent polarity has a key role in the recovery furano-flavonoids – pongamol and karanjin – from the seeds of *P. pinnata*. As both these active compounds don't have any polar characteristics, extraction with polar solvents exhibited low recovery and liposoluble properties of these phytochemicals increases the recovery by non-polar solvents like hexane.



Journal of Pharmaceutical Research and Innovation (JPRI)
Volume 6, Issue 2, July 2026
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Figure 3. Effect of different extraction solvents on karanjin contents is extracts (Data are presented as mean ± standard deviation of the mean (n = 4)).

Effect of Extraction Solvents on Free Radical Scavenging Activity

Antioxidant activity by DPPH analysis is mainly reported as IC_{50} and this is the minimum concentration of antioxidant molecules required to scavenge 50% of the DPPH free radicals present initially in the system.

Table 2 summarizes the antioxidant activity of *P. pinnata* seed extract made by different solvent systems. It was observed that extract obtained from hexane extraction showed an average IC_{50} value of 36.9 mg/ml and this is the lowest among all other extraction systems. This pointed out the high radical scavenging ability of hexane extract in comparison with the other solvent systems. Acetone extract and ethanol extract showed 38.9 and 41.3 mg/ml respectively whereas water extract had the highest value of 132.8 mg/ml which confirmed the lowest antioxidant activity.

Table 2. Effect of different extraction solvents on radical scavenging

Solvent	DPPH IC_{50} (mg/mL)				Average
	Trial 1	Trial 2	Trial 3	Trial 4	
Hexane	37.2	36.5	36.8	37.2	36.9
Acetone	38.5	38.1	39.2	39.8	38.9
Ethanol	40.7	41.3	42.7	40.5	41.3
Water	133.2	130.2	135.2	132.4	132.8

Effect of Extraction Methodologies on Oil Yield

Effect of different extraction methodologies on the yield of extraction with the same solvent hexane from *P. pinnata* seeds depicted in "Figure 4". From the data it was observed that Soxhlet extraction brings highest yield (25.0%) whereas hot extraction (22.9%) and maceration (21.9%) showed notably less yield. When compared to the lowest to highest yields it was noticed that Soxhlet extraction provide approx. 14% hike in the extraction yield. The noteworthy hike in the extraction yield by Soxhlet extraction due to the increased number of fresh washes in this extraction methodology.

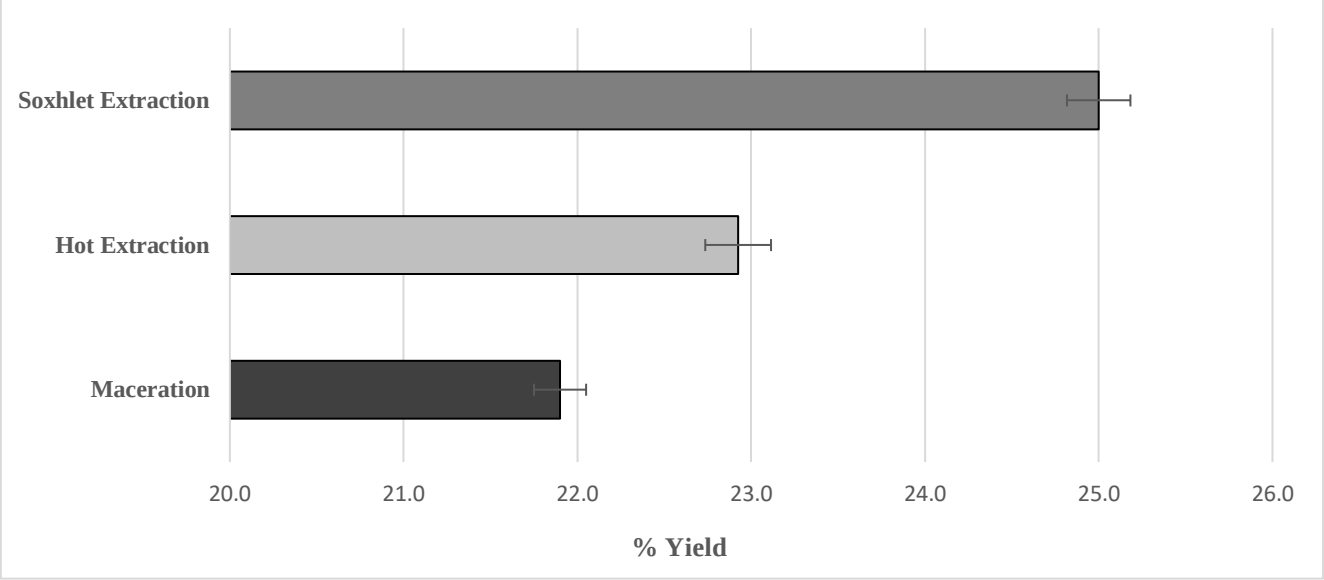

Figure 4. Effect of different extraction methods on extraction yields from *P. pinnata* seeds (Data are presented as mean ± standard deviation of the mean (n = 4))

Effect of Extraction Methodologies on Furano Flavonoids Contents

Effect of different extraction methods on the recovery of pongamol showed in "Figure 5". It ws observed that Soxhlet could provide higher pongamol content (0.38%) and hot extraction showed a pongamol content of 0.34% whereas maceration brought 0.32% pongamol content to the extract. There were 18% hike in the pongamol content using Soxhlet extraction when comparing with maceration and this is a similar pattern as shown in the increment in the extraction yield.

Journal of Pharmaceutical Research and Innovation (JPRI)
Volume 6, Issue 2, July 2026
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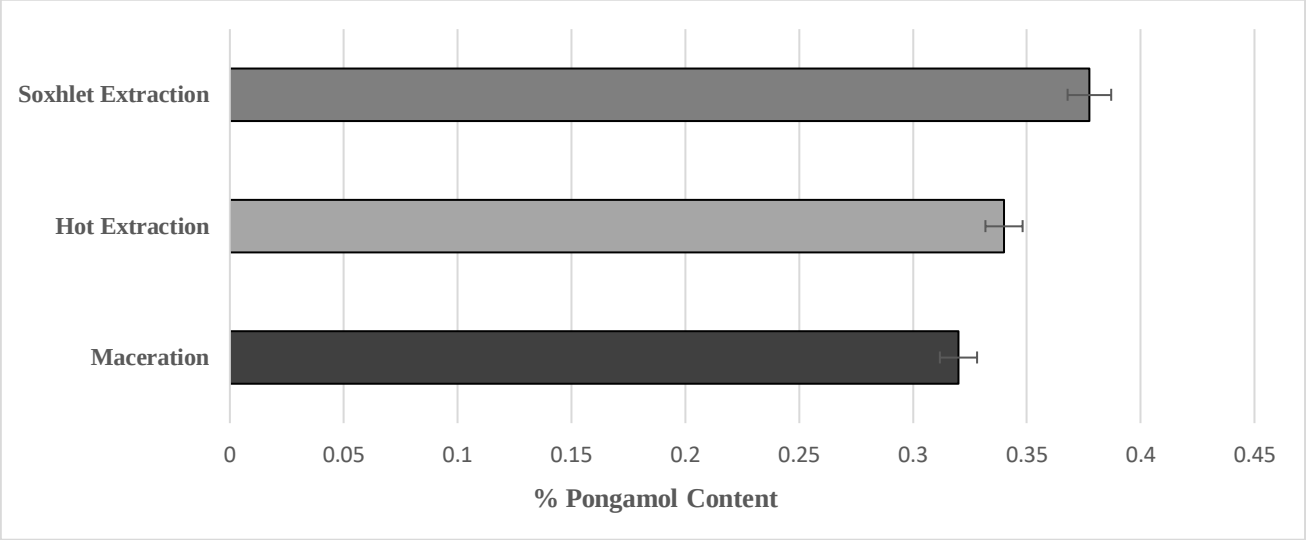

Figure 5. Effect of different extraction methods on pongamol contents in extracts (Data are presented as mean ± standard deviation of the mean (n = 4)).

Karanjin content in the seed extracts obtained by different extraction methods summarized in "Figure 6". Seed extract by Soxhlet extraction contains 2.1% karanjin content whereas hot extraction and maceration provide seed extract with 1.82% and 1.70% karanjin content respectively. Comparison of maceration and Soxhlet with respect to the karanjin content of the seed extract showed a 23% hike with the latter.

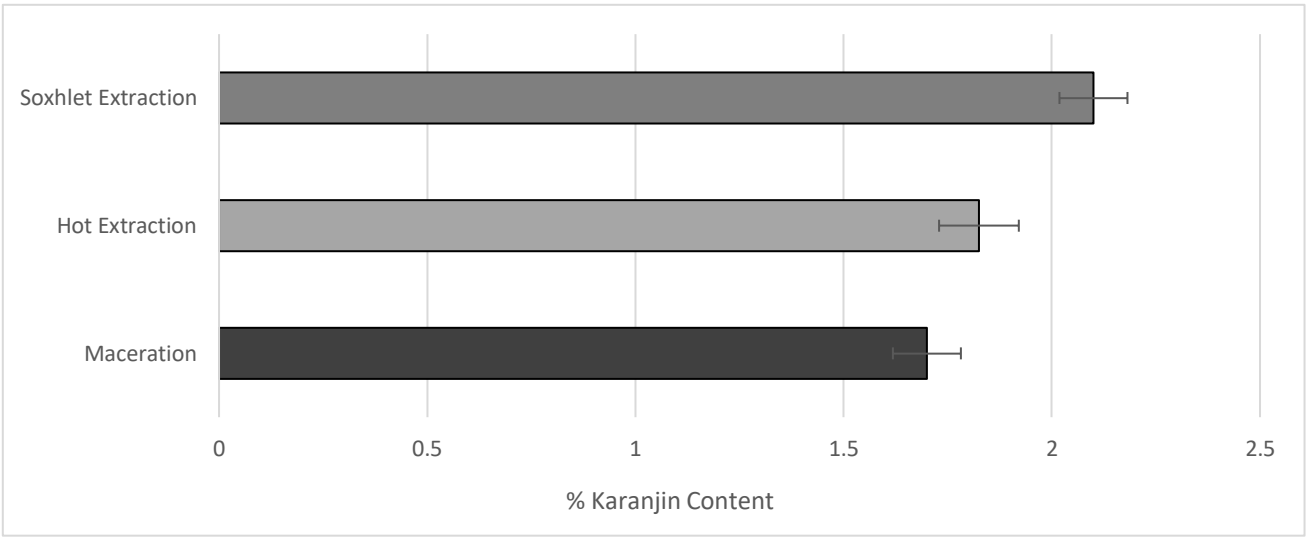

Figure 6. Effect of different extraction methods on karanjin content (Data are presented as mean ± standard deviation of the mean (n = 4))

Effect of Extraction Methods on Free Radical Scavenging Activity

Antioxidant activity of the seed extract obtained by different extraction methodologies shown in Table 3. Extract made by Soxhlet extraction showed lowest IC_{50} value of 33.7 mg/ml and hence with highest free radical scavenging activity. Extract with hot extraction showed 35.2 mg/ml and extraction by maceration showed 36.9 mg/ml IC_{50} values. The radical scavenging activity data is in compliance with the data observed by Rekha et al. (2020) [15].

Table 3. Effect of different methods on radical scavenging

Solvent	DPPH IC_{50} (mg/mL)				Average
	Trial 1	Trial 2	Trial 3	Trial 4	
Maceration	37.2	36.5	36.8	37.2	36.9

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Hot Extraction	35.2	35.8	34.9	34.8	35.2
Soxhlet Extraction	34.3	34.5	33.9	32.1	33.7

Anticancer Activity

Anti skin cancer studies using human skin cancer cells (A375) bring promising and consistent results as this is one of the most widely used human cutaneous melanoma cell lines. The predictable growth characteristics of these cell lanes open up opportunities to evaluate the anti-cancer effectiveness of molecules in research.

Effectiveness of the prevention of skin cancer cell lane A 375 along with the *P. pinnata* seed extract made by Soxhlet hexane extraction shown in "Figure. 7". The figure shows the microscopic images of A375 cells treated with different concentrations of *P. pinnata* seed extract.

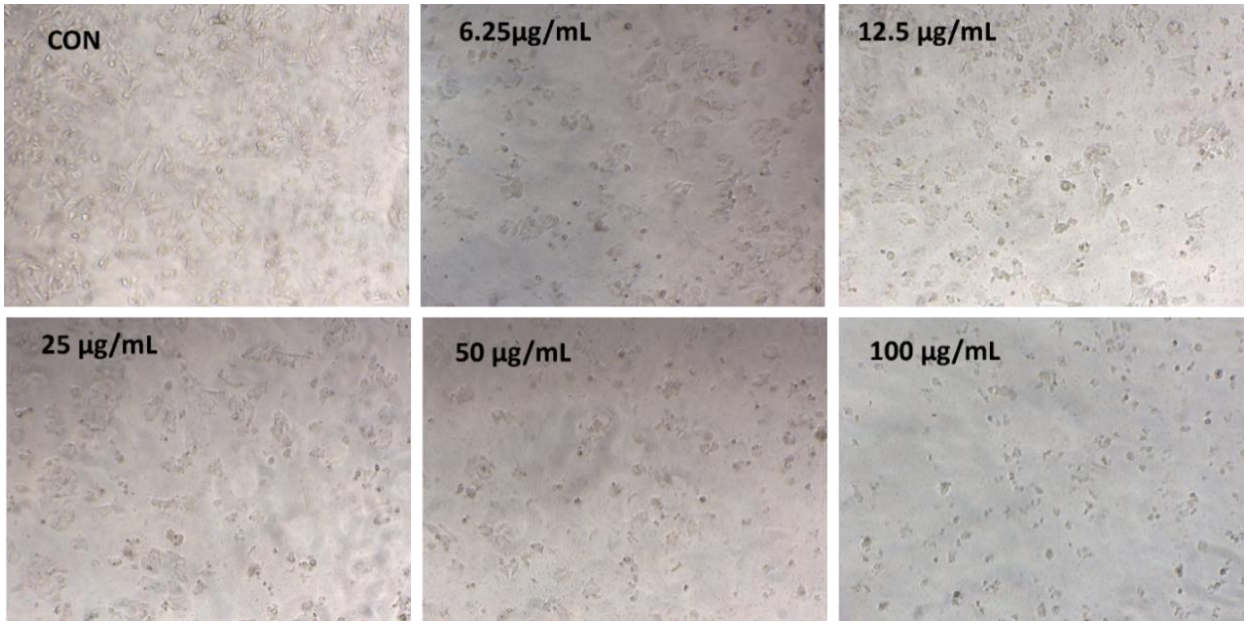

Figure 7. Anti-cancer effectiveness of *P. pinnata* seed extract on A375 skin cancer cells

The inhibitory response of the extract across different concentrations is presented in "Figure. 8". The extract exhibit loss dose-dependency towards the cytotoxicity, with the highest inhibition of 24% observed at 25 µg/mL. Activity increased gradually up to this concentration, after which a sudden change in response was noted, possibly due to antagonistic interactions among coextracted phytochemicals. Linear increment in the inhibition of cancer cell growth against concentration of seed extract was observed up to 25 µg/mL level indicates that there is potential in the seed extract as an anti-skin cancer ingredient and if the active phytochemical compounds isolated from the *P. pinnata* seeds there will be much higher activity.

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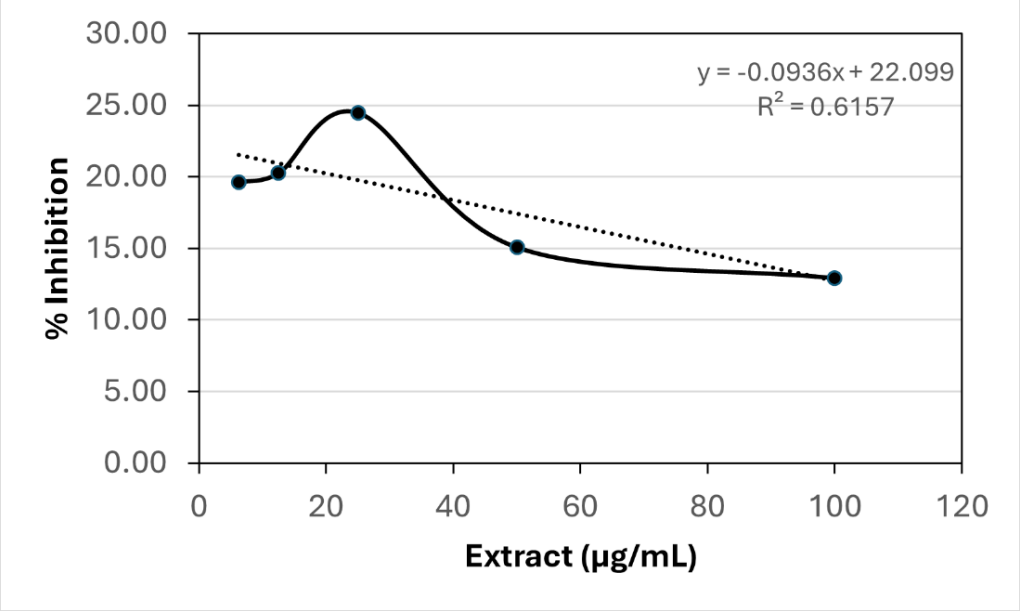

Figure 8. Anti-cancer effectiveness of extract by graphical representation

CONCLUSION

Current study evaluates the effect of solvent polarity on the extraction yield and recovery of furano-flavanoids from *P. pinnata* seeds. The major phytochemicals from *P. pinnata* seeds are liposoluble in nature and hexane seemed to be the best choice of extraction in comparison with acetone, ethanol and water. This confirms the effectiveness of polarity of the extraction solvent on the recovery of active compounds from the raw material. Impact of extraction solvent also had impact on the antioxidant activity of the extract obtained and hexane provided extract with higher antioxidant activity as well. Current study also evaluated different extraction methodologies with hexane as extraction solvent. Among the different extraction methods, Soxhlet extraction proved to be the best technique for the extraction of *P. pinnata* seeds in comparison with maceration and hot extraction. Soxhlet extraction provides the highest yield, highest furano-flovanoid contents, and lowest IC_{50} value, hence the highest antioxidant activity. The current study further evaluates the anti-skin cancer activity of *P. pinnata* seed extract obtained by Soxhlet extraction with hexane. The seed extract showed 24% inhibition of skin cancer cell growth with a concentration of 25 µg/mL. There was linear hike in the inhibition till 25 µg/mL and this showed the potential of this extract against skin cancer. Future course of work needs to be the evaluation of anti-skin cancer activity of isolated furanoflavanoids – pongamol and karanjin - as it seems that there will be higher activity.

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