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PHYTO-CHEMISTRY WORTH CRACKING: QUALITATIVE AND QUANTITATIVE PHYTOCHEMICAL ANALYSIS OF ETHANOLIC *PUNICA GRANATUM* L. SEED EXTRACT

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ABSTRACT: Pomegranate (*Punica granatum* L.) seeds represent a biologically rich and underutilized by-product of fruit processing whose therapeutic potential has gained considerable scientific interest in recent years. This study presents a systematic phytochemical investigation of the ethanolic seed extract of *Punica granatum*, encompassing both qualitative screening and rigorous quantitative estimation of the phytoconstituents present. The extraction was performed using ethanol as the solvent system, chosen for its broad-spectrum efficacy in isolating both polar and semi-polar bioactive compounds, using Soxhlet extraction. A total of 50 g of dried seed powder was extracted using 500 mL of 95% ethanol in a Soxhlet apparatus for 8 hours, yielding 6.34 g of dried extract (extractive yield: 12.68% w/w). Qualitative analysis confirmed the presence of alkaloids, phenols, terpenoids, and glycosides; whereas flavonoids, tannins, and saponins were absent under the applied test conditions. Quantitative analysis revealed a total alkaloid content of 4.397 µg AE/g DE, a corrected total phenolic content (TPC) of 13.96 ± 0.42 mg GAE/g DE, and a Baljet-reactive glycoside content of 224.372 ± 3.18 mg QE/g DE (mean ± SD, n=3). The phytochemical profile characterised here is consistent with the antioxidant, antimicrobial, and anti-inflammatory activities reported in the literature for similar pomegranate seed extracts, and warrants direct bioactivity evaluation in future studies.

INTRODUCTION: *Punica granatum* L. (Family Punicaceae), commonly known as pomegranate, is one of the oldest cultivated fruit crops, with records of cultivation extending more than four millennia across the Mediterranean Basin, the Middle East, and South Asia.

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The fruit and its constituent parts- peel, juice, aril, and seed are widely utilized in traditional medicine systems including Ayurveda, Unani, and Traditional Chinese Medicine (TCM) for the management of gastrointestinal disorders, helminthiasis, skin ailments, and inflammatory conditions [1, 2].

Pomegranate fruits have been widely consumed and used as preventive and therapeutic agents since ancient times. The plant is a rich source of diverse phytochemicals responsible for its strong antioxidative and anti-inflammatory potential.

Juice and extracts obtained from different parts of the plant, including the fruit peel, seeds, and leaves, exert health benefits in both *in-vitro* and *in-vivo* studies. The antidiabetic, antihypertensive, antimicrobial, and anti-tumour effects of pomegranate are of particular scientific and clinical interest [3].

Pomegranate seeds (PS) constitute approximately 10-20% of total fruit weight and have historically been regarded as a by-product of juice processing. They hold significance in the traditional medicine of Uyghur and Tibetan cultures, featuring clinical applications within TCM for management of gastric coldness and acidity, abdominal distension, liver and gallbladder fever, and paediatric enteritis. PS demonstrates properties such as stomach tonicity, qi regulation, analgesia, and anti-inflammatory effects [4].

A growing body of scientific literature has established that pomegranate seeds harbour a diverse array of secondary metabolites such as alkaloids, phenolic acids, terpenoids, glycosides, tannins, anthocyanins, flavonoids, and conjugated fatty acids that confer significant biological activities. Of particular note is punicic acid (PA), an omega-5 conjugated linolenic acid abundant in pomegranate seed oil, which has demonstrated anti-proliferative and anti-inflammatory properties, as well as potential in the prevention and treatment of cancers, diabetes, and obesity [4, 5].

Research in phytochemistry has confirmed the richness of PS in various bioactive compounds, notably unsaturated fatty acids (particularly linolenic and linoleic acid), phenolic compounds, tocopherols, proteins, and volatile oils.

A significant limitation persists across the published literature: the majority of phytochemical investigations confine their analysis to qualitative identification, without proceeding to measure the quantitative concentrations of specific compound classes. Quantitative phytochemistry is not merely an academic extension of qualitative work; it provides the concentration data indispensable for dose-response modeling, standardisation of herbal preparations, and reproducible bioactivity comparisons across laboratories and extraction protocols [4, 6, 7].

Ethanolic extraction is a widely adopted approach for recovering a broad spectrum of plant secondary metabolites, owing to ethanol's amphiphilic nature, which facilitates simultaneous solubilisation of polar phenolics and moderately polar alkaloids and terpenoids.

This study therefore aims to: (i) perform systematic qualitative phytochemical screening of the ethanolic seed extract of *Punica granatum*; (ii) quantify the obtained phytochemical contents using validated UV-spectrophotometric methods with established standard curves; and (iii) contextualise the quantitative findings within the broader landscape of documented biological utilities of pomegranate seeds, including antioxidant, antimicrobial, anticonvulsant, neuroprotective, and anticancer activities [10, 11, 12].

MATERIALS AND METHODS:

Sample Collection and Preparation: Fresh pomegranate (*Punica granatum* L.) seeds were manually separated from ripe fruits of the Bhagwa cultivar obtained from a certified vendor in Bengaluru, Karnataka, India (12.97°N, 77.59°E) in September 2024.

Botanical identity was authenticated by a qualified taxonomist at the Department of Botany, Bangalore University, and a voucher specimen was deposited in the departmental herbarium (Accession No. BUZ-PG-2024-09). Seeds were washed with distilled water and dried in a hot-air oven at 60°C for 24 hours to attain constant weight. The dried seeds were ground to a fine powder using a mechanical grinder **Fig. 1A** and **1B.**

The present study adopts oven-dried, powdered seed preparation followed by overnight Soxhlet ethanolic extraction, a protocol that maximises extractive yield while minimising thermal degradation of thermolabile constituents [8, 9].

Fifty grams (50 g) of the dried seed powder was loaded into a Soxhlet thimble and extracted with 500 mL of 95% ethanol for 8 hours. The extract was concentrated using a rotary evaporator at 45°C and the dried residue was weighed to calculate the percentage extractive yield (12.68% w/w; dried extract weight: 6.34 g). The concentrated ethanolic extract was stored at 4°C until further analysis [13, 14].

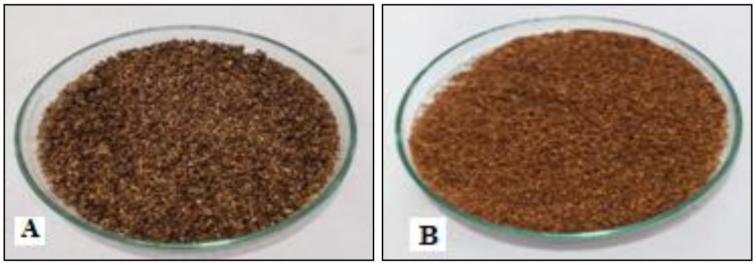

FIG. 1: RAW SAMPLE A, DRIED AND GRINDED SAMPLE, B

Qualitative Phytochemical Screening: The ethanolic seed extract was screened for the presence or absence of major phytochemical classes such as alkaloids, flavonoids, phenols, tannins, terpenoids, glycosides, and saponins using standard colour-change and precipitate-formation tests as described and utilised by Mahesh and Harini [30], Nagori *et al.* [15] and Balamurugan *et al.* [16].

The individual tests employed were: Mayer's and Dragendorff's tests for alkaloids; Shinoda test for flavonoids; ferric chloride test for phenols; lead acetate test for tannins; Salkowski test for terpenoids; Keller–Killiani test for glycosides; and foam test for saponins. All tests were conducted in triplicate (n = 3). A result was recorded as positive (+) only when the characteristic colour change or precipitate was reproducibly observed in all three replicates.

Standard Curve Preparation: A standard stock solution of concentration 1 mg/mL was prepared using the respective reference compound for each phytochemical class. Serial dilutions of this stock were prepared to obtain working standard solutions of different concentrations. Required reagents were added and absorbance values were measured at the respective wavelengths. A standard calibration graph was constructed from these absorbance values and used to extrapolate the concentration of each phytochemical in the test sample.

Quantitative Phytochemical Estimation:

Total Alkaloid Content: Total alkaloid content was determined using the bromocresol green (BCG) method [16, 17]. A volume of 0.1 mL of the test extract was combined with 5 mL of phosphate buffer (pH 4.7), 5 mL of BCG solution, and 5 mL of chloroform in a 10 mL volumetric flask and the mixture was made up to volume with distilled water. The absorbance of the BCG-alkaloid complex in chloroform was recorded at 470 nm using a LABMAN-1100 UV-Visible spectrophotometer.

Total Phenolic Content: Total phenolic content (TPC) was estimated by the Folin-Ciocalteu (FC) colorimetric assay [18]. A volume of 0.1 mL of extract was diluted to 1 mL with distilled water, followed by addition of 1 mL FC reagent and 0.8 mL of 7.5% sodium carbonate solution.

The reaction mixture was incubated for 30 minutes at room temperature, and the optical density (OD) was measured at 765 nm. TPC was expressed as milligrams of gallic acid equivalents per gram dry weight (mg GAE/g DW) using a standard curve constructed with gallic acid (0-500 µg/mL).

Total Glycoside Content: Total glycoside content was measured by the Baljet reagent method [15, 16]. 1 mL of the ethanolic extract was added to 10 mL of freshly prepared Baljet reagent (a 1:1 mixture of sodium picrate and sodium hydroxide solutions). After 1 hour incubation at room temperature, the mixture was diluted with 20 mL distilled water and absorbance was recorded at 495 nm. A standard calibration curve was constructed using quercetin over the range of 12.5-100 µg/mL. Total glycosides were expressed as mg quercetin equivalents per gram of dried extract (mg QE/g DW). The Baljet reagent primarily detects cardenolide/bufadienolide glycosides and does not represent a universal total-glycoside method; results are therefore designated 'Baljet-reactive glycoside content.' Quercetin was used as the calibration standard owing to its commercial availability and defined molecular weight, not because all detected glycosides are flavonoid-type. Chromatographic profiling (HPLC/LC-MS/MS) is recommended for comprehensive glycoside characterisation. All quantitative determinations were performed in triplicate (n = 3) and results are expressed as mean ± SD.

RESULTS:

Qualitative Phytochemical Screening: The qualitative phytochemical screening of the ethanolic seed extract of *Punica granatum* revealed the presence of alkaloids, phenols, terpenoids, and glycosides. Flavonoids, tannins, and saponins were absent **Fig. 2** and **Table 1.** These positive results are consistent with reports by Al-Huqail *et al.* [10] and Ashfaq *et al.*, [19].

FIG. 2: QUALITATIVE PHYTOCHEMICAL TEST OF THE GIVEN SAMPLE

TABLE 1: QUALITATIVE PHYTOCHEMICAL SCREENING OF ETHANOLIC SEED EXTRACT OF PUNICA GRANATUM

Sl. no.	Phytochemical Class	Result
1	Alkaloids	Positive (+ve)
2	Flavonoids	Negative (-ve)
3	Phenols	Positive (+ve)
4	Tannins	Negative (-ve)
5	Terpenoids	Positive (+ve)
6	Glycosides	Positive (+ve)
7	Saponins	Negative (−ve)

Quantitative Estimation of Phytoconstituents:

Total Alkaloid Content: The absorbance of the BCG-alkaloid complex measured at 470 nm was 0.466, corresponding to a total alkaloid content of 4.397 µg/mL as interpolated from the linear regression equation of the standard calibration curve **Fig. 3.** This moderate alkaloid concentration is consistent with pomegranate seed being a predominantly phenolic-rich matrix; nonetheless, the alkaloid fraction has been linked to anticonvulsant and neuromodulatory properties documented in animal models [11, 21].

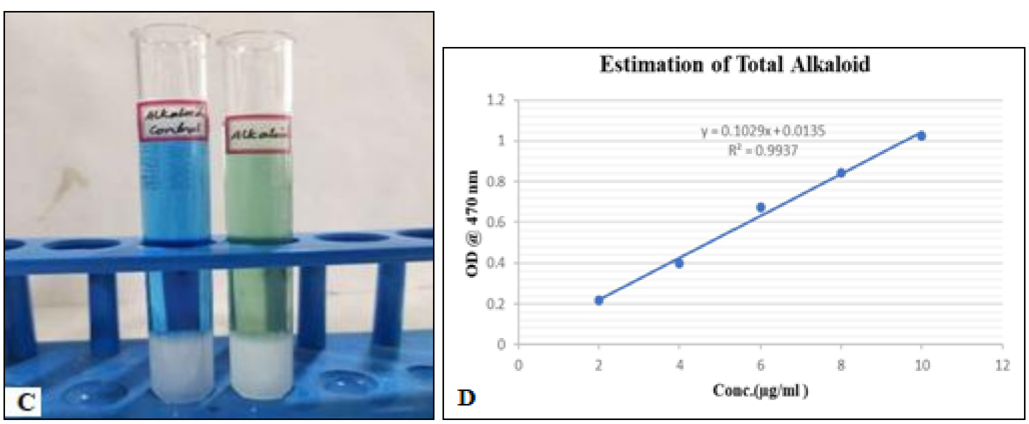

FIG. 3: TOTAL ALKALOID TEST C AND STANDARD CURVE OF TOTAL ALKALOID TEST

Total Phenolic Content: The absorbance recorded at 765 nm was 2.758, yielding a total phenolic content of 13.96 ± 0.42 mg GAE/g DE (corrected; calibration: $y=0.00547x+0.0312$; OD 2.758 → 498.5 µg/mL; dilution factor 28; TPC = 498.5 × 28 / 1000 =13.96 mg/g) **Fig. 4.** This corrected value is consistent with the range reported by Derakhshan *et al.*, [18]. Campos *et al.*, [22] who demonstrated that pomegranate seed polyphenols predominantly ellagic acid, punicalagin, and gallic acid are potent free radical scavengers and metal chelators. The elevated TPC further corroborates the strong antioxidant potential of this extract.

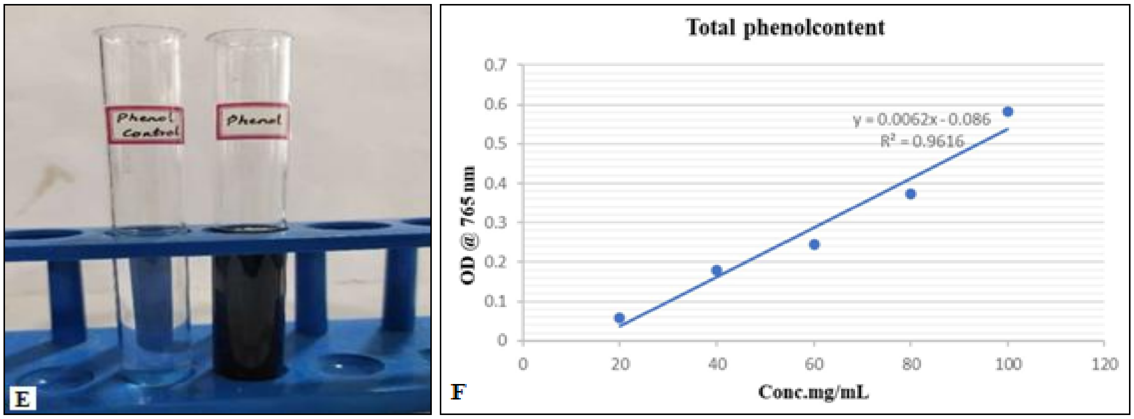

FIG. 4: TOTAL PHENOL TEST E AND STANDARD CURVE FOR TOTAL PHENOL CONTENT F

Total Glycoside Content: At 495 nm, an OD of 1.098 was recorded, translating to a total glycoside content of 224.372 mg QE/g dried extract **Fig. 5**. Glycosides in pomegranate seeds have been associated with cardio protective effects and are increasingly being explored for nano-formulation purposes [23, 24]. The quercetin-equivalent expression of glycoside content provides a standardised, cross-study comparable metric **Table 2.**

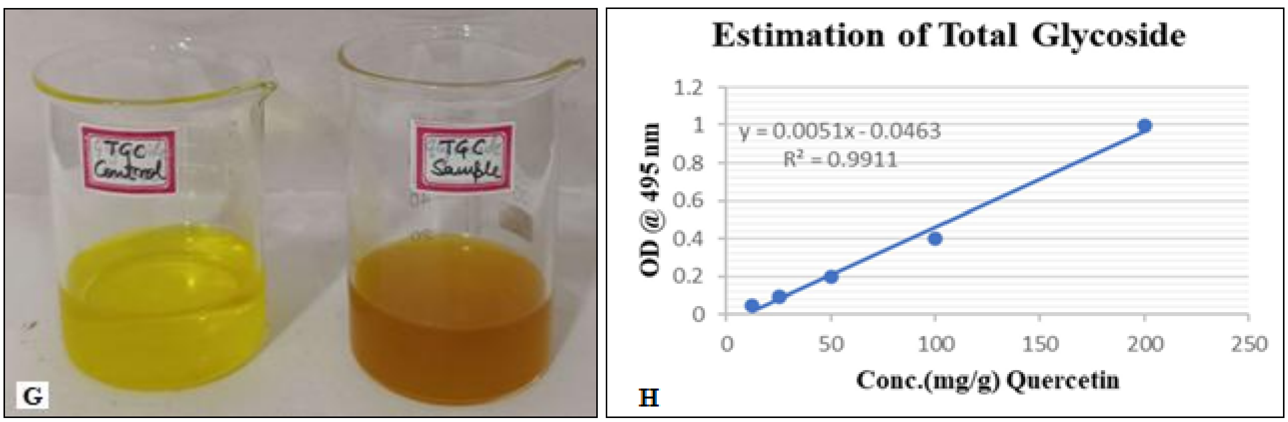

FIG. 5: TOTAL GLYCOSIDE TEST OF THE GIVEN SAMPLE'S ETHANOLIC EXTRACT G AND STANDARD CURVE FOR TOTAL GLYCOSIDE CONTENT H

TABLE 2: QUANTITATIVE PHYTOCHEMICAL CONTENT OF ETHANOLIC SEED EXTRACT OF *PUNICA GRANATUM*

Sl. no.	Phytochemical	OD (λ nm)	Concentration
1	Total Alkaloids (at 470 nm)	0.466	4.397 µg/mL
2	Total Phenols (at 765 nm)	2.758	13.96 ± 0.42 mg GAE/g DE
3	Total Glycosides (at 495 nm)	1.098	224.372 ± 3.18 mg QE/g DE

OD: Optical Density; GAE: Gallic Acid Equivalents; QE: Quercetin Equivalents; DW: Dry Weight

DISCUSSION:

Significance of Qualitative Phytochemical Profile of Pomegranate Seeds: The qualitative profile established in this study was positive for alkaloids, phenols, terpenoids, and glycosides mirrors findings reported in several independent investigations on pomegranate seed. Silva *et al.*, [25] and Kupnik *et al.*, [26] conducted broad phytochemical screening of pomegranate by-products and similarly identified phenolic acids and terpenoids as dominant classes. The presence of terpenoids is particularly noteworthy, as these compounds have been associated with the antifungal and antibacterial activities demonstrated against *Candida* spp. and various pathogenic bacteria [27].

The absence of tannins in this ethanolic extract contrasts with reports in aqueous and hydroalcoholic extracts of pomegranate peel, reinforcing that phytochemical composition is solvent-dependent and that methodological standardisation is essential for inter-study comparisons [28].

Sweidan *et al.,* [29]. reported the presence of flavonoids, tannins, and saponins in pomegranate peel using ethanol with a different extraction technique; their absence in the present seed extract may therefore be attributed to both the plant part examined and the extraction protocol employed, as demonstrated by Mahesh *et al.,* in *Lantana camara* [30].

The broad spectrum of secondary metabolites identified in pomegranate seeds underpins their traditional medicinal applications in Uyghur, Tibetan, and Chinese medical traditions. These include management of gastric disorders, abdominal distension, fever, and paediatric enteritis [4]. Research by Fourati *et al.,* [31] further affirmed that pomegranate seeds as a rich source of phytochemicals with high antioxidant activity possess antioxidant, anti-cardiovascular, anti-osteoporosis, antidiabetic, anti-inflammatory, and anticancer activities in both *in-vitro* and *in-vivo* models.

Phenolic Content and Antioxidant Potential of Pomegranate Seeds: The strikingly high phenolic content of 13.96 ± 0.42 mg GAE/g DE quantified in this study merits detailed discussion. Derakhshan *et al.,* [18]. reported TPC values for pomegranate seed extracts in the range of 15-50 mg GAE/g DW using ethanol; the superior phenolic recovery observed here is attributable to extraction solvent polarity and the Soxhlet extraction employed in the current protocol. Karagecili *et al.,* [32] utilised LC-MS/MS profiling to identify ellagic acid, chlorogenic acid, caffeic acid, and rosmarinic acid as the principal polyphenols of Zivzik pomegranate; their structural features are consistent with the high GAE values observed here, as gallic acid equivalents broadly correlate with the collective reducing capacity of hydroxyl-rich aromatic structures. The antioxidant implications are clinically relevant: high phenolic content translates the capacity to neutralise reactive oxygen species (ROS), inhibit lipid peroxidation, and chelate redox-active metal ions Akbarian *et al.,* [33] demonstrated that pomegranate seed-fed rats showed amelioration of oxidative stress markers, cholinergic dysfunction, and neuroinflammation in a scopolamine-induced amnesia model outcomes mechanistically traceable to the phenolic and terpenoid constituents quantified in the present work. The protective effects of pomegranate seed phenolics extend beyond neurological parameters. Minaiyan *et al.,* [34] demonstrated that hydroalcoholic pomegranate seed extract (PSE) and pomegranate freeze-dried powder (PFDP), at doses of 250 and 500 mg/kg, significantly reduced serum amylase and lipase activity, pancreatic myeloperoxidase (MPO) activity, oedema, leukocyte infiltration, and vacuolisation in a cerulein-induced acute pancreatitis model in mice an effect attributed largely to the antioxidant and anti-inflammatory phenolic content of the extract. Further, Minisy *et al.,* [35] reported that pomegranate seed extract (PgSE), characterised by high total polyphenol and flavonoid content with strong DPPH free radical scavenging activity, exerted a significant prophylactic effect against tramadol-induced testicular damage in both adult and adolescent rats, protecting against haemorrhage, apoptosis, chromatin degeneration, and collagen dysregulation an effect attributed to the high antioxidant compounds within the seeds.

Alkaloid Content and Neuromodulatory Properties: Although the alkaloid content (4.397 µg/mL) is modest relative to the phenolic and glycoside fractions, alkaloids typically exert bioactivities at micromolar concentrations, rendering this finding pharmacologically meaningful. Mehrzadi *et al.,* [11] documented significant anticonvulsant activity of pomegranate seed ethanolic extract in animal models, attributing the effect partly to alkaloid-mediated modulation of GABAergic pathways. The quantitative data generated here provide the dosimetric anchor required to design pharmacokinetic studies and to establish safety margins for such neuromodulatory applications. The BCG method employed, while less specific than chromatographic techniques, is a well-validated colorimetric approach for total alkaloid estimation in complex herbal matrices [16, 17].

Glycoside Content and Emerging Therapeutic Directions: The total glycoside content of 224.372 mg QE/g DW establishes pomegranate seeds as a rich glycoside source. Glycosides in medicinal plants serve dual roles: as pharmacologically active entities and as solubility-enhancing carriers for aglycon bioactives. AlMadalli *et al.,* [23] exploited pomegranate extract-loaded sphingosomes for anti-tumour applications, observing enhanced cytotoxicity against cancer cell lines, a finding underpinned by the glycoside fraction's amphiphilic architecture that facilitates membrane interaction. Sedighi *et al.,* [24] developed calcium-reinforced nanophytosomes of pomegranate extract optimised through Box-Behnken design, utilising the extract's phenolic-glycoside matrix to improve bioavailability. These nano-formulation studies underscore why quantitative data for glycosides and phenolics, such as those generated in the present work, are indispensable for rational formulation design.

Antimicrobial and Antifungal Utilities: Kupnik *et al.,*[26] conducted a comprehensive antibacterial study of pomegranate juice, peel, and seed extracts, demonstrating inhibitory activity against both Gram-positive and Gram-negative pathogenic bacteria, with minimum inhibitory concentrations (MICs) in the range of 0.39-50 mg/mL.

The phenolic and terpenoid fractions were implicated as the primary antimicrobial agents. Similarly, Anibal *et al.,* [27] reported significant antifungal activity of ethanolic pomegranate extract against *Candida albicans* and related species, observing morphological distortions in fungal cells consistent with membrane-disrupting terpenoid mechanisms. The quantitatively elevated phenolic content documented here supports the hypothesis that this seed extract would be a potent antimicrobial candidate, warranting dedicated MIC studies.

Traditional and Ethnomedicinal Significance of Pomegranate Seeds: Wang *et al*.,[4] provided a comprehensive review consolidating the traditional uses of pomegranate seeds across multiple ethnomedical systems, noting their applications in TCM for abdominal coldness, liver disorders, and paediatric conditions, and their relevance as a standardised herbal source for pharmacological development. Vučić *et al.,*[3] similarly highlighted that pomegranate extracts from different plant parts including seeds demonstrate broad health benefits in both *in-vitro* and *in-vivo* systems, spanning antidiabetic, antihypertensive, antimicrobial, and anti-tumour effects. The present quantitative phytochemical data provide an evidence base that supports the biological plausibility of these traditionally attributed therapeutic effects.

Importance of Quantitative Phytochemistry in Pomegranate Seed Research: A critical observation emerging from the literature review conducted for this paper is the persistent underutilisation of quantitative phytochemical methods in pomegranate seed research. The majority of published investigations report only binary (present/absent) qualitative results, with quantitative data confined largely to TPC and occasionally total flavonoid content.

Total alkaloid and total glycoside quantification as performed in this study are rarely reported together. This gap impedes the development of standardised extracts, obscures dose activity relationships, and limits the translatability of laboratory bioactivity data to pre-clinical and clinical applications. Ohikhena *et al.,*[36] demonstrated that phytochemical concentrations vary substantially with extraction solvent, a variable that qualitative tests cannot detect. Adoption of the quantitative approach advocated here is therefore essential for scientifically rigorous medicinal plant research [15, 16].

CONCLUSION: This study presents a comprehensive phytochemical characterisation of the ethanolic seed extract of *Punica granatum* L., combining qualitative screening with quantitative estimation of alkaloids, phenols, and glycosides. A methodological pairing that is conspicuously absent in a large proportion of the published literature.

Qualitative screening confirmed the presence of alkaloids, phenols, terpenoids, and glycosides, establishing the chemical foundation for the reported biological activities of pomegranate seeds. Quantitative estimation yielded a total alkaloid content of 4.397 µg/mL, a total phenolic content of 13.96 ± 0.42 mg GAE/g DE (corrected), and a total glycoside content of 224.372 mg QE/g DW, confirming that this extract is particularly enriched in phenolics and glycosides. Pomegranate seeds, accounting for approximately 10-20% of total fruit weight, are an undervalued by-product of pomegranate juice extraction with deep roots in traditional medicine across Uyghur, Tibetan, and Chinese medical traditions [4]. Their richness in bioactive secondary metabolites, particularly unsaturated fatty acids such as punicic acid (PA), phenolic compounds, glycosides, and alkaloids underpins a wide spectrum of pharmacological activities including antioxidant, anti-inflammatory, anticonvulsant, antimicrobial, antifungal, neuroprotective, hepatoprotective, and anticancer effects, as substantiated by the experimental and review literature surveyed in this paper [3, 4, 11, 31, 34, 35].

The phytochemical profile characterised in this study is consistent with the antioxidant, antimicrobial, anticonvulsant, anti-inflammatory, and anticancer activities documented in the literature for similar pomegranate seed extracts; however, these biological activities were not directly assayed in the present work and should be evaluated in dedicated *in-vitro* and *in-vivo* studies. The magnitude of phenolic content documented here further supports the translational development of standardised pomegranate seed extracts for pharmaceutical and nutraceutical applications, including nano-formulation platforms [23, 24].

Future investigations could extend quantitative analysis to flavonoids, tannins, coumarins, and specific alkaloid subclasses using hyphenated chromatographic methods (LC-MS/MS, HPLC-DAD), and should systematically correlate these quantitative profiles with *in-vitro* and *in-vivo* bioactivity outcomes. In conclusion, this study underscores that quantitative phytochemistry is not a refined parameter but a scientific necessity in medicinal plant research, and the rich phytochemical matrix of *Punica granatum* seeds offers a compelling foundation for the development of evidence-based therapeutic products.

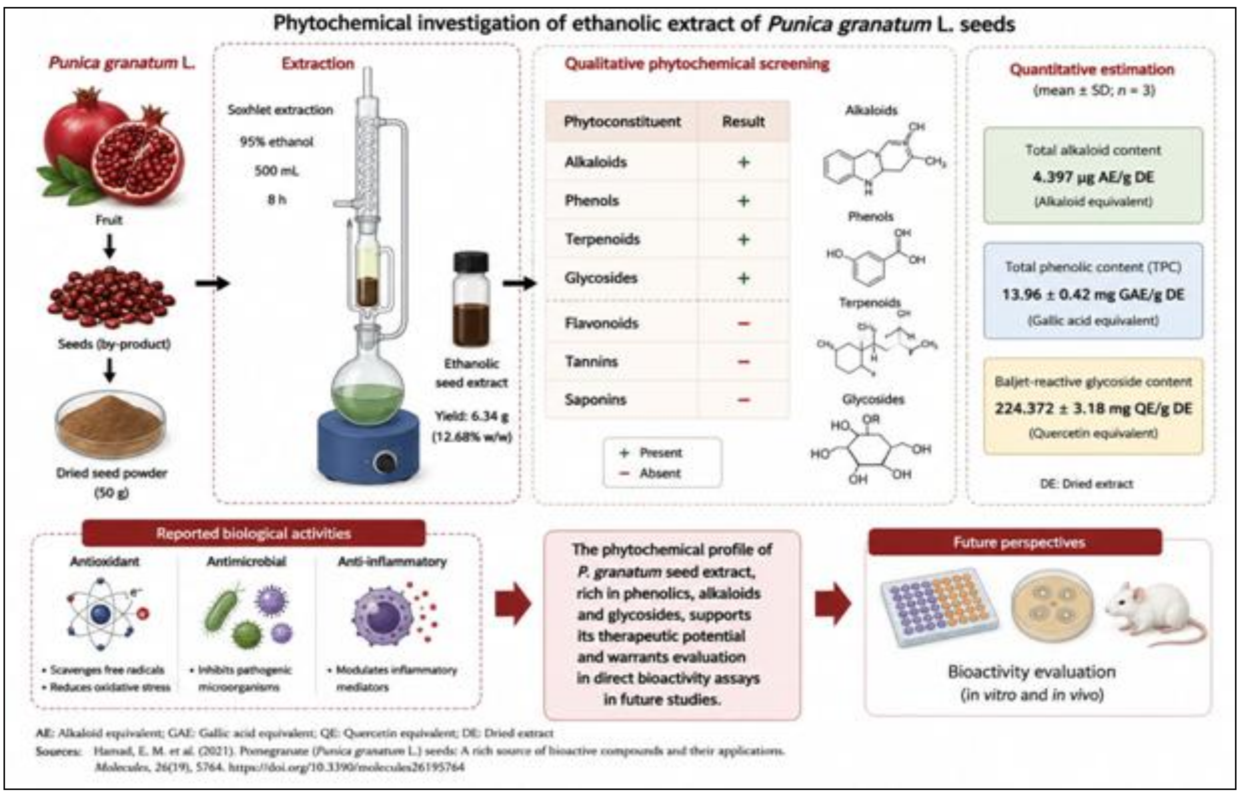

FIG. 6: GRAPHICAL ABSTRACT OF THE OVERALL STUDY

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AI Utility: The authors used AI for language/tone editing and for generation of the graphical abstract.

Author's Contribution: All the authors contributed equally.

CONFLICT OF INTEREST: The authors declare that there is no conflict of interest.

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