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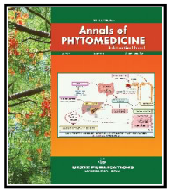

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Influence of plant matrix, solvent polarity and extraction technique on phytochemical profile and *in vitro* biological activity of *Ziziphus nummularia* (Burm. f.) Wight and Arn. grown in Rafha, Saudi Arabia

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Abstract

The current research evaluates the influence of plant matrix, solvents and methods of extraction on phytoconstituents and *in vitro* anti-inflammatory activities of *Ziziphus nummularia* (Burm. f.) Wight and Arn. (*Z. nummularia*) seeds and leaves. Methanol, chloroform and petroleum ether were used as solvents. The methods used for extraction were maceration (ME), Soxhlet (SE) and microwave-assisted extraction (MWE). Proteinase inhibitory and albumin denaturation activity were utilised to access the anti-inflammatory properties. The results reveal that plant matrix, solvent and the method of extraction play an important role in the quantity of phytoconstituents, which in turn affect biological activities. Use of green technologies like MWE, led to the highest total phenolic content (TPC) (630.66 ± 2.8 GAE/g) and total flavonoid content (TFC) (142.66 ± 2.8 QE/g). Methanol proved to be the best solvent. Concerning the plant matrix, the leaves were found to be richer in these constituents as compared to the seeds.

The methanol leaf extract obtained from the MWE method offered the maximum anti-inflammatory activity at 500 µg/ml in the albumin denaturation as well as the proteinase inhibitory method. The percentage inhibition of protein inhibition was 88.79 % at 500 µg/ml, while proteinase inhibitory activity was found to be 73.83% at the same concentration. In conclusion, the best method for extraction of polyphenols from the leaves was found to be MWE using methanol as a solvent. This study provides useful insight into the extraction conditions (technique, solvent, and plant matrix) for the evaluation of anti-inflammatory property.

1. Introduction

Secondary plant constituents have been traditionally used for healing human disorders all over the globe across different cultures and civilisations from time immemorial. The identification and characterization of these bioactive compounds from medicinal plant matrices require diligent choice of suitable extraction methods and solvents (Azwanida, 2015). The yield, quantity and medicinal activity are determined by plant matrix, techniques and solvent used for extraction. Solvents of varying polarities are used for extracting bioactive compounds. Inexpensive, convenient, well-planned and highly relevant extraction technology is desirable for phytochemical exploration (Morais *et al*., 2016). The main aim of extraction is to obtain the maximum quantity of secondary metabolites of importance. The use of a single solvent or method for extraction is not possible due to the diversity of phytoconstituents. Therefore, the extraction of bioactive constituents has always remained a problem for researchers. The nature of the polarity of the compound is important for the selection of the solvent and the method chosen for extraction. Furthermore, the extraction of yield also varies with different extraction methods (Shailesh *et al*., 2022; Arawande *et al*., 2021). Different solvents of varying polarities have been used either individually or in combination for effective extraction of phytoconstituents. The outcome was different with different methods used for extraction, which was baffling for the investigators (Suman *et al*., 2022; Sasidharan *et al*., 2011). Hence, it is pertinent to find out the most appropriate solvent and method for the extraction of these plant metabolites. Several methods are available for the extraction of plant materials, of which solvent extraction is commonly used. Literature reveals that the size, plant part used, solvent, stage of development, extraction temperature and time will affect the efficiency of extraction (Suprada *et al*., 2023; Insha *et al*., 2022; Patel *et al*., 2019). Therefore, it is difficult to predict the appropriate extraction solvent for different plant parts. Hence, this research was done with the aim of investigating the solvent and method for extraction of the phytoconstituents and also to document the effect of this on the *in vitro* anti-inflammatory activity of *Z. nummularia* as most research on this plant is focused on the evaluation and identification of active compounds. As per our understanding, we have not come across any study that has published the influence of matrix, solvent and extraction methods on the phytochemicals and biological activity of this plant.

Z. nummularia (Family: Rhamnaceae) is a thorny bush that grows in dry arid areas. Leaves are usually ovate but sometimes elliptic with

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a round base. Flowers are 4-5 mm, the calyx lobes are acute, the petals obovate or rounded. The ovary is two-locules. The different parts of the plant are traditionally used as food, and to treat constipation, skin diseases, conjunctivitis, diarrhoea, helminthiasis., *etc.* (Mesmar *et al*., 2022). The reported biological activities are antioxidant, antimicrobial, antiproliferative, hypoglycemic and hypolipidemic, sedative-hypnotic, antipyretic, anticancer and anthelmintic (Kumar *et al*., 2010; Bodroth and Das, 2012; Ray and Dewanjee, 2015). Some of the major phytoconstituest present are nummularine, nummularine-P, nummularine-S, amphibin-H, jubanine-A-B, mauritine-C and nummularogenin (Tschesche *et al*., 1975; Miana and Shah, 1985; Shah *et al*., 1989).

2. Materials and Methods

2.1 Collection of plant materials

Leaves and seeds of *Z. nummularia* were hand-picked from Rafha, Saudi Arabia. They were washed to remove any adhering material and dried in the shade. The leaves and seeds were pulverized separately using an electrical blender and were sieved. The crude sample was stored in airtight containers in a standard laboratory environment. The sample was preserved with the voucher specimen (PC-2023-2) in the Faculty of Pharmacy, Northern Border University for future studies.

2.2 Preparation of extracts

Each of the samples was extracted separately with each solvent of varying polarities, *i.e.*, petroleum ether, chloroform and methanol. Three methods were used for extraction; maceration, Soxhlet and microwave-assisted solvent extraction (Nortjie *et al*., 2022). Maceration extraction (ME): The powders were suspended in each solvent separately at room temperature for 20 h in a container and were stirred at intervals. Soxhlet extraction (SE): The powders were extracted in 250 ml of each solvent separately using a Soxhlet extractor. Microwave-assisted extraction (MWE): The powders were extracted in 100 ml of each solvent separately for 10 min with intermittent cooling in a Microwave oven (model: TM-38W, input 220V-50-60HZ,1500W, size 38 L).

The extracts thus obtained were filtered and concentrated. The percentage yield of the extracts was calculated using the formula:

$$\text{Percentage yield} = \frac{\text{weight of the extract}}{\text{weight of the sample}} \times 100$$

2.3 Preliminary phytochemical screening

Screening of the extracts was carried out for flavonoids, alkaloids, phenolic compounds, tannins, *etc*., in accordance with standard methods (Shah and Quadry, 1995).

2.4 Estimation of total phenolic content, total flavonoids and condensed tannins

Total phenolic content (TPC), total flavonoid content (TFC) and condensed tannins (CT) were determined spectroscopically using an APEL/PD-303UV spectrophotometer (Alshrari *et al.,* 2020; Monika *et al*., 2023). The TPC was calculated by following the Folin-Ciocalteu method and absorbance was recorded at 750 nm. It was expressed as µg per gram of gallic acid equivalent (GAE). The calibration curve for standard gallic acid was plotted using suitable dilutions. TFC was calculated using the colourimetric assay of aluminum chloride and absorbance was measured at 415 nm. It was expressed as quercetin equivalent per gram (QE/g). Condensed tannins were evaluated using vanillin assay using a working reagent of 1% vanillin and 8% concentrated HCl. The absorbance was recorded at 500 nm. The CT was calculated as the catechin equivalent of the calibration curve of standard catechin.

2.5 Anti-inflammatory activity

In vitro anti-inflammatory activity was evaluated by the following methods (Sakat *et al.,* 2010; Naira *et al*., 2019).

2.5.1 Inhibition of albumin denaturation

Solutions comprising varying concentrations of the extracts and 1% solution of bovine albumin were prepared and pH adjusted with 1N hydrochloric acid. This was followed by heating of the samples at 57°C. The turbidity was assessed at 660 nm using an APEL/PD-303UV spectrophotometer.

2.5.2 Proteinase inhibitory effect

Trypsin (0.06 mg), hydrochloric acid buffer (1ml), and a sample of 1ml methanol and chloroform extract of various concentrations were prepared and incubated for 5 min. To this mixture, 1 ml of 0.8% casein was incorporated, followed by the addition of 2 ml of perchloric acid. The absorbance was recorded at 210 nm. The following formula was used for calculating the percentage inhibition:

$$\%\ \text{Inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

All experiments for evaluation of TPC, TFC, CT and *in vitro* anti-inflammatory activity were conducted in triplicate. The values were depicted as mean ± standard deviation. Comparison between groups was done with a student's t-test. Probability values < 0.05 were treated as significant.

3. Results

3.1 Qualitative phytochemical screening

Qualitative screening of the seeds and leaves of *Z. nummularia* using different solvents and various methods of extraction was carried out. The seeds and leaves in all methods of extraction using methanol and chloroform contain phenolics, flavonoids and sterols. The results of the preliminary qualitative screening are presented in Table 1.

3.2 Effect of solvent, method of extraction and plant matrix on yield and phytochemical composition

The results of the present study exhibited that the extraction yield was dependent on the nature of the solvent and extraction method. The yields of extracts varied significantly, ranging from 26.2% to 3.4% based on extraction method, plant matrix and solvent. Among the solvents, the highest yield was obtained from methanol (leaves 26.2%, seeds 12.4%), followed by chloroform (leaves 18.0%, seeds 10.3%) and petroleum ether (leaves 8.6%, seeds 3.4%). The yield of the extract was highest in the MWE.

The contents of TPC, TFC and TC for petroleum ether, chloroform and methanol of both leaf and seed extracts varied. The TPC in the leaves was found to be in the range of 630.66 ± 2.8 and 60.66 ± 0.53

mg GAE/g, while in the seeds, it was 541.33 ± 2.3 and 46.33 ± 1.4 mg GAE/g, which was determined from the linear regression equation (y = 0.1308x + 0.0298, R^2 = 0.9921) as shown in Figure 1. The TPC with reference to different solvents used for extraction were as follows: methanol > chloroform > petroleum ether. The TPC was highest in the extracts obtained from MWE. However, the leaves were richer in phenols and flavonoids when compared to the seeds. The results of TPC are depicted in Figure 2.

Table 1: Phytochemical screening of the leaves and seeds in various solvents and method of extraction

Extraction methods/ solvents used/plant matrix	Phytoconstituents											
	Alkaloids		Glycosides		Phenolics		Flavonoids		Sterols		Fixed oils	
Maceration Parts of the plant	Leaf	Seed	Leaf	Seed	Leaf	Seed	Leaf	Seed	Leaf	Seed	Leaf	Seed
Petroleum ether	-	-	-	-	-	-	+	+	+	+	+	+
Chloroform	-	-	-	-	+	+	+	+	+	+	+	+
Methanol	+	+	+	+	+	+	+	+	+	+	-	-
Soxhlet												
Petroleum ether	-	-	-	-	-	-	+	+	+	+	+	+
Chloroform	-	-	-	-	+	+	+	+	+	+	-	-
Methanol	+	+	+	+	+	+	+	+	+	+	-	-
Microwave-assisted solvent extraction												
Petroleum ether	-	-	-	-	-	-	-	-	+	+	+	+
Chloroform	-	-	-	-	+	+	+	+	+	+	+	+
Methanol	+	+	+	+	+	+	+	+	+	+	+	+

(+) indicates the presence of phytochemicals, (-) indicates the absence of phytochemicals.

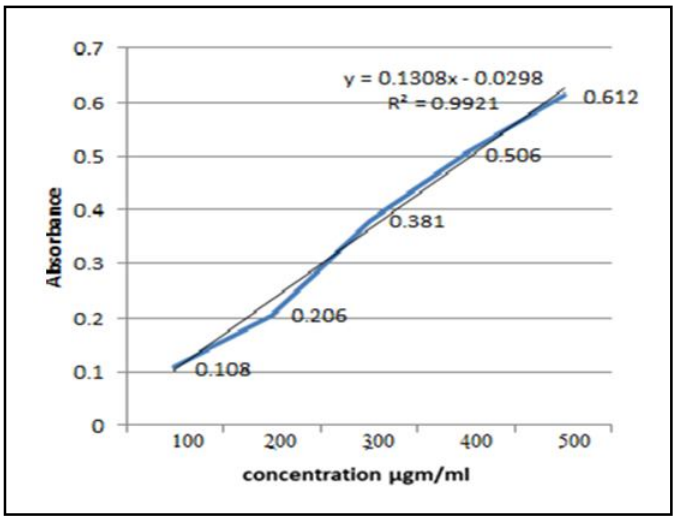

Figure 1: Calibration curve for standard gallic acid.

Results of the TFC were determined from the linear regression equation (y = 0.133x + 0.269, R^2 = 0.9933) is shown in Figure 3. The maximum amount of TFC was obtained from the methanolic extracts of the leaves. However, methanolic leaf extract obtained from MWE gave the highest yield (142.66 ± 2.8 mg QE/g), while the least yield was obtained with SE (97.66 ± 2.9 mg QE/g) as represented in Figure 4, which may be the result of oxidation and degradation of compounds due to exposure to higher temperature and the longer duration of extraction time.

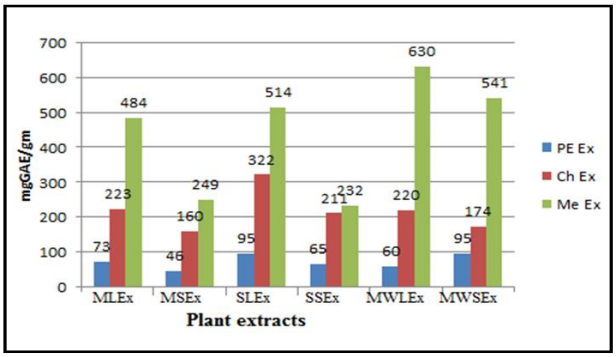

Figure 2: Total phenolic content of *Z. nummularia* extracts.

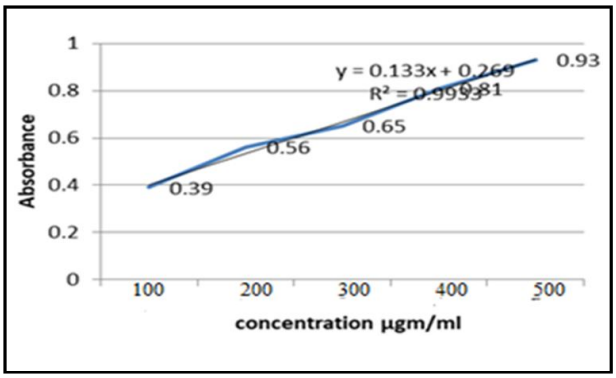

Figure 3: Calibration curve of standard quercetin.

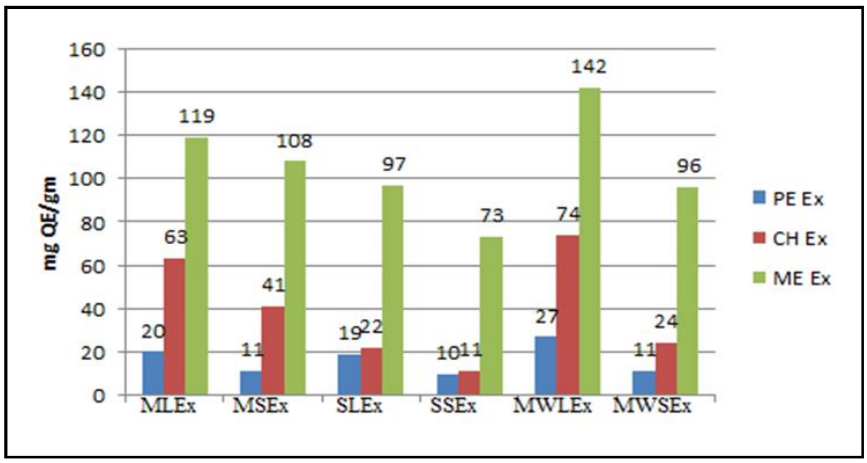

Figure 4: Total flavonoid content of *Z. nummularia* extracts.

The TPC and TFC in increasing order were: MWE > ME > SE. From our results, it is evident that the plant matrix, solvent used, and extraction process have a notable influence on the plant phytochemicals.

Results of the CT were evaluated from the linear regression equation obtained for catechin ($y = 0.089x-0.001$, $R^2 = 0.9961$) as depicted in Figure 5. The amount of CT was in the range of 43.66 ± 0.5 and 246 ± 0.9 mg mg CT/gm, depending on the plant matrix, solvent and method of extraction. The highest amounts were found in the methanolic extracts of the leaves. However, the method of extraction did not significantly affect its quantity. Figure 6 represents the CT content of the extracts.

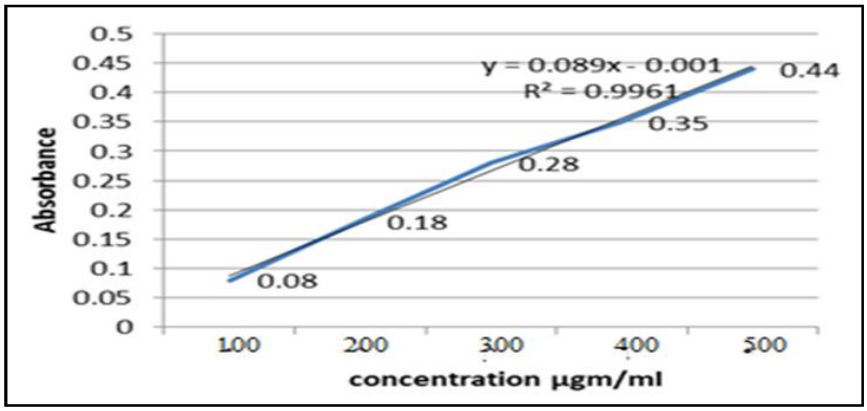

Figure 5: Calibration curve for standard catechin.

PE: petroleum extract, CH: chloroform extract, ME: methanolic extract, MLEx: leaf extract obtained by maceration, MSEx: seed extract obtained by maceration, SLEx: leaf extract obtained by soxhlet extraction, SSEx: seed extract obtained by Soxhlet extraction, MWLEx: leaf extract obtained by microwave-assisted extraction, MWSEx: seed extract obtained by microwave-assisted extraction.

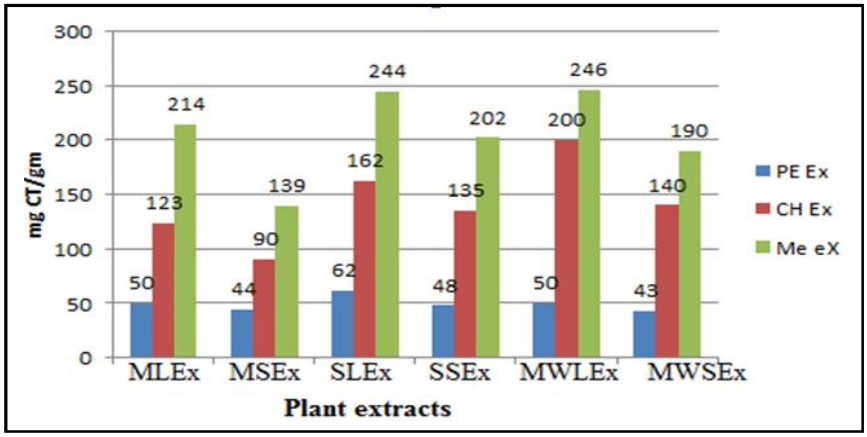

Figure 6: Condensed tannin content of *Z. nummularia* extracts.

3.5 Effect on *In vitro* anti-inflammatory activity

Two methods were used to gauge the activity, *i.e.,* inhibition of albumin denaturation assay and proteinase inhibitory effect. Petroleum ether extract was not used for this study as the amount of TPC, TFC and CT was less. The extracts of all the tested medicinal plant materials possessed anti-inflammatory activity by inhibiting protein denaturation in the range of 88.79% to 46.46% at 500 µg/ml. In the present study, the standard used was diclofenac sodium, and it exhibited a maximum inhibition of 89.81% at 500 µg/ml (IC_{50} 6.38 µg/ml). Among the extracts, the methanolic leaf extract obtained from the MWE offered the maximum anti-inflammatory activity in both the leaves (88.79%, IC_{50} 12.45 µg/ml, $p<0.05$) and seeds (75.22%, IC_{50} 42.30 µg/ml) at of 500 µg/ml. The suppression of protein denaturation was dose-dependent. The albumin denaturation activity of the extracts using different plant parts, matrices, solvents and methods is depicted in Figures 7 and 8.

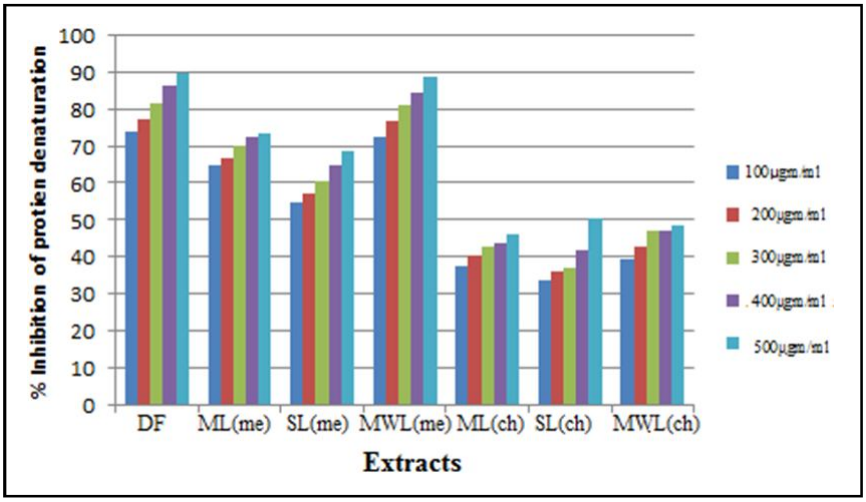

Figure 7: Effect of *Z. nummularia* leaf extract on albumin denaturation.

DF-standard diclofenac, ML (me) methanol leaf extract obtained by maceration, SL (me) methanol leaf extract obtained by Soxhlet extraction, MWL (me) methanol leaf extract obtained by microwave-assisted extraction, ML(ch) chloroform leaf extract obtained by maceration, SL (ch) chloroform leaf extract obtained by soxhlet extraction, MWL (ch) chloroform leaf extract obtained by microwave-assisted extraction.

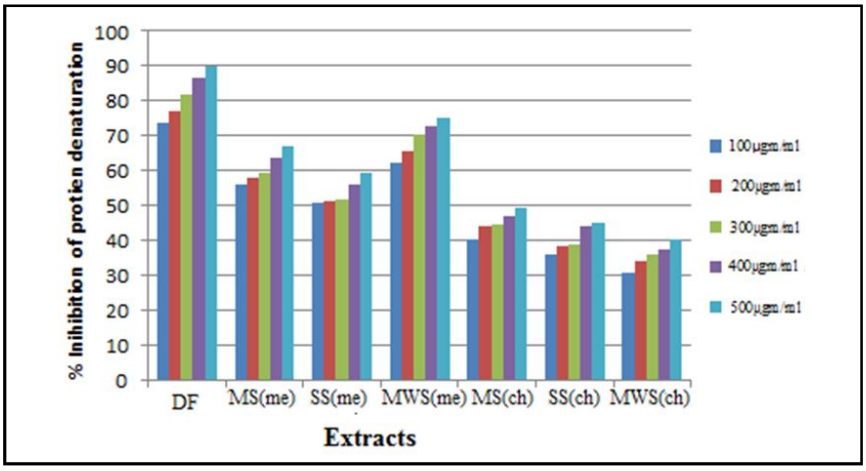

Figure 8: Effect of *Z. nummularia* seed extract on albumin denaturation.

DF-standard Diclofenac, MS(me) methanol seed extract obtained by maceration, SS(me) methanol seed extract obtained by Soxhlet extraction, MWS(me) methanol seed extract obtained by microwave-assisted extraction, MS(ch) chloroform seed extract obtained by maceration, SS(ch) chloroform seed extract obtained by Soxhlet extraction, MWS(ch)chloroform seed extract obtained by microwave-assisted extraction.

In the proteinase inhibition method, the standard drug ibuprofen showed maximum inhibition of 84.46 ± 0.26 IC_{50} 23.91 µg/ml, at 500 µg/ml. The activity of the extracts varied in the range from 73.83 ± 0.00 to 24.57 ± 0.10 in proteinase inhibitory activity at 500 µg/ml. The MWE offered the maximum anti-inflammatory activity in the leaves (73.83%) and the seeds (50.41%) at 500 µg/ml. This inhibition was attributed to the amount of TPC and TFC in the methanolic extract of the leaves and seeds. The anti-inflammatory activities with regard to different methods of extraction used were as follows: microwave > Soxhlet > maceration in these methods. The anti-protease activity of extracts at various concentrations in the present study is shown in Figures 9 and 10.

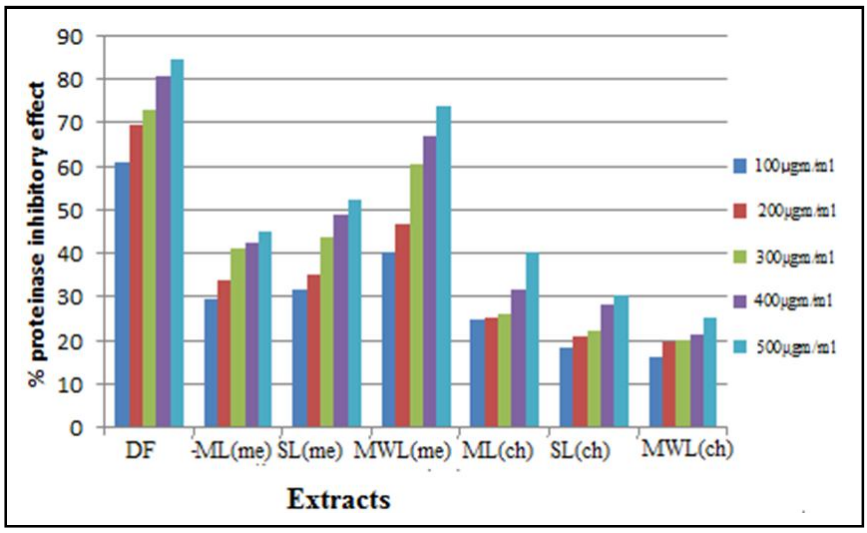

Figure 9: Effect of *Z. nummularia* leaf extract on proteinase inhibition.

ML(me) methanol leaf extract obtained by maceration, SL (me) methanol leaf extract obtained by Soxhlet extraction, MWL (me) methanol leaf extract obtained by microwave-assisted extraction, ML (ch) chloroform leaf extract obtained by maceration, SL(ch) chloroform leaf extract obtained by Soxhlet extraction, MWL(ch) chloroform leaf extract obtained by microwave-assisted extraction.

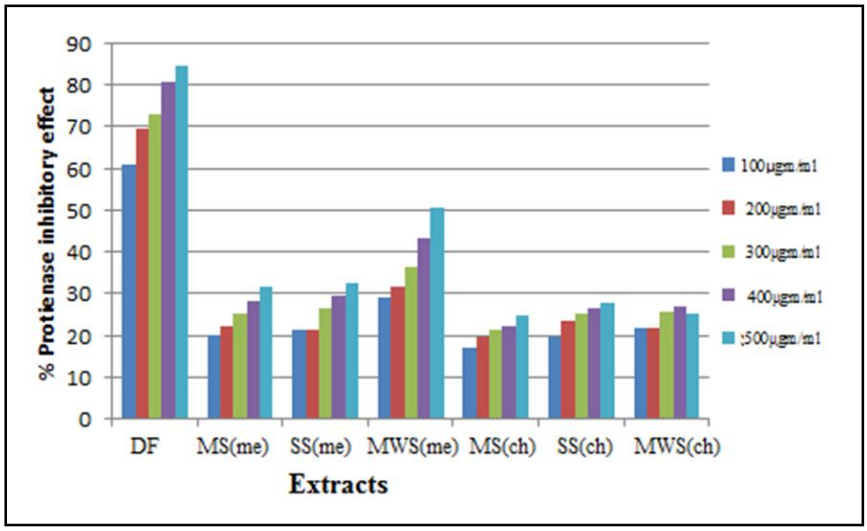

Figure 10: Effect of *Z. nummularia* seed extract on proteinase inhibition.

MS(me) methanol seed extract obtained by maceration, SS(me) methanol seed extract obtained by Soxhlet extraction, MWS(me) methanol seed extract obtained by microwave-assisted extraction, MS(ch) chloroform seed extract obtained by maceration, SS(ch) chloroform seed extract obtained by Soxhlet extraction, MWS(ch) chloroform seed extract obtained by microwave-assisted extraction.

4. Discussion

The findings of this study give us valuable information about the best solvent and the method of extraction for the evaluation of phenolic compounds from this plant. Extraction is one of the most significant steps to recover and identify active compounds in plants. The efficiency of extraction is altered by the extraction method and time, temperature, chemical nature and solvent polarity (Turkmen *et al*., 2006). In our study, polar constituents were well extracted in methanol. This indicates that the extraction favours polar solvents as the polar components (phenols, glycosides, and flavonoids) are extracted by polar solvent. Our results are similar to the available literature wherein maximum extract yield is obtained with polar solvents (Sultana *et al*., 2009; Anwar *et al*., 2006). Furthermore, the extraction yield is not only influenced by the solvent but also by the method used for extraction. Irrespective of the solvent used, the MWE method was found to have a higher yield over the other two methods, which may be due to the faster diffusion of the solute from the plant cells into solvent due to heat and pressure impact on cell walls (Dhanani *et al*., 2017). MWE is an innovative solvent extraction technology that has some advantages such as; lesser solvent is consumed, time duration is shortened, recovery rate is high and is cost-effective (Afoakwah *et al*., 2012; Rama *et al*., 2023).

Polyphenolic, like phenolic acids, flavonoids and condensed tannins are secondary metabolites found ubiquitously in plants which act as biologically active compounds, possessing anti-inflammatory

properties (Ali Reza *et al*., 2023; Asdaq *et al*., 2008). Hence, the content of these three classes of phytoconstituents was evaluated. The results of our study revealed that these phytoconstituents were more in the methanolic extracts, indicating that maximum extraction of polyphenolics is generally obtained from the polar solvent as it possesses better solvation as a result of interactions between the polar sites of the compounds and the solvent when compared to the nonpolar solvents (Thouri *et al*., 2017; Harborne, 1999). Therefore, methanol is an efficient solvent commonly used for the extraction of polyphenols. An ideal solvent for extraction is characterized by its extraction and capacity for conserving the chemical structure of desired compounds. The amount of polyphenolics present in the MWE was more when compared to Soxhlet extraction. This may be because of oxidation and degradation of compounds due to exposure to higher temperatures and the longer duration of extraction time**.** The TPC and TFC in increasing order were: MWE > ME > SE. Thereby giving evidence that the MWE method is a better option for obtaining the extraction of secondary plant metabolites. This is supported by the available literature (Sultana *et al*., 2009; Stojievi *et al*., 2018). Similarly, the amount of these constituents was more in the leaves when compared to the seeds. From our results, it is evident that the plant matrix, solvent used, and extraction process have a notable influence on the phytoconstituents of this plant.

Anti-inflammatory activity was determined by denaturation of protein and proteinase inhibition. Denaturing of proteins can cause loss/modification of function and may produce autoantigens that can be a contributing factor to inflammation. Albumin denaturation is one of the most common methods used to probe the anti-inflammatory potential of plant extracts as it is reliable and sensitive. Literature reports that alteration of albumin proteins gives rise to the generation of antigens which in turn initiate type III hypersensitive reaction causing inflammation; therefore, inhibition of denaturation by any extract suggests anti-inflammatory properties (Modi *et al*., 2019). Literature reports the impact of various plant matrices on protein denaturation (Kumar *et al*., 2013; Govindappa *et al*., 2011). Methanol extracts exhibited higher anti-inflammatory activity when compared to chloroform extracts. The extracts with higher levels of total phenolics exhibited greater anti-inflammatory activity, showing a correlation between activity and content of phenolics. Proteinases play a vital role in arthritic reactions. Literature reports that leukocyte proteinase may have a significant role in tissue damage during the various inflammatory stages and defence is provided by proteinase inhibitors (Govindappa *et al*., 2011). The results of our research revealed that there was a correlation between the studied anti-inflammatory properties and phenolic content. Various recent studies have shown that phenolic compounds like phenolic acids and flavonoids exhibit anti-inflammatory activities by virtue of their ability to scavenge free radicles (Gunathilake *et al*., 2018; Deekshitha *et al*., 2023). This indicates that the evaluated biological activity may be attributed to the presence and synergistic activity of polyphenols, flavonoids, and other phytoconstituents.

5. Conclusion

The chemistry of secondary metabolites varies; hence different plant matrices, solvents and methods of extraction may have different effects on solubility, extraction yield and biological activity. The results of our study indicate that the microwave-assisted solvent extraction method is the best extraction method and can be a viable alternative to traditional extraction methods. This study provides useful insight into the extraction conditions (technique, solvent, and plant matrix) for the evaluation of the anti-inflammatory property of *Z. nummularia*. However, further studies are required to optimize the various parameters of the extraction process. Green extraction techniques like microwave-assisted extraction would be able to save energy, and time and improve the yield.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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