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***Research Article** (Open Access)*

SCREENING OF THE *IN VITRO* ANTI-INFLAMMATORY EFFICACY OF *ANISOMELES INDICA (L)* WHOLE PLANT EXTRACTS BY ALBUMIN DENATURATION TECHNIQUE AND EVALUATION OF IT’S TOTAL PHENOL, ALKALOID AND FLAVONOID CONTENTS

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ABSTRACT

The anti-inflammatory potential of *Anisomeles indica* (Linn) (Lamiaceae) entire plant was assessed in this research by employing the (*in vitro*) albumin denaturation assay .The screening was performed in its various solvent extracts , namely ethyl acetate, methanol, and aqueous extracts . Total flavonoid (TFC) and Total phenolic (TPC) contents of the crude extracts were estimated by aluminium chloride and the Folin-Ciocalteu method, respectively. The reaction of alkaloid with bromocresol green formed the basis for the quantification of alkaloids in the sample extracts. The percentage of inhibition of albumin denaturation was dose-dependent. Methanol extract demonstrated the highest protection against denaturation (81.04%) (IC50: 26.44 g/mL), followed by the aqueous extract (76.10%) and then ethyl acetate extract (28.25%). Ibuprofen, the reference drug, had the highest inhibition rate of 88.73%. Highest quantities of phenols (148.5 mg GAE/g) and flavonoids (133.55 mg Quercetin/g), were observed in the methanol extract, indicating a positive correlation to the extract’s anti-inflammatory potency and thereby validating the plant's pharmacological significance.

Keywords: *Anisomeles indica, Albumin denaturation, Anti-inflammatory activity, Phenol, Alkaloid, Flavonoid*

INTRODUCTION

The majority of people, especially in underdeveloped nations, count on herbal extracts to satisfy most fundamental medical requirements (Hosseinzadeh *et al.,* 2015). For several decades, plant metabolites have been recognized and explored as potential sources for novel therapeutics instead of the synthetic compounds, as they are safe with less side effects and minimal toxicity (Rahman *et al.,* 2021). The ability to chelate metal ions, quench free radicals and singlet oxygen(Boora *et al.,* 2014),have made the bioactive molecules (Flavonoids, phenols, and alkaloids)(Taylor *et al.,* 2001) from plants as potent tools to combat the complications caused by free radical damage and inflammation (Nanda *et al.,* 2007; Truong *et al.,* 2019). *Anisomeles indica* (Linn) commonly called Catmint belongs to the Lamiaceae/Labiatae family (Kirtikar and Basu., 1999). It is widely prevalent in the tropical regions of the world including India, and South East Asia (Antil *et al.,* 2019).This ethno -medically important shrub is aromatic in nature and is used to manage an array of illnesses (Nasrin *et al.,* 2019).Previous research has indicated that the roots (Yusuf *et al.,* 1994) possess anti –inflammatory whereas the whole plant extracts have demonstrated anti- microbial activity , capacity to inhibit cell proliferation and inflammation (Wang *et al.,* 2005; Hsieh *et al.,* 2008; Rao *et al.,* 2009; Huang *et al.,* 2012) .Further studies on leaves and stem have proven it’s free radical scavenging ability and cyclooxygenase -1 inhibiting potency (Dharmasiri *et al.,* 2002). The essential oils from the leaves is effective against rheumatoid arthritis when applied externally (Nasrin *et al.,* 2019). The current research seeks to investigate the protein

protecting efficacy of various solvent preparations from the *Anisomeles indica Kuntze (Linn)* whole plant using the inhibition of albumin denaturation in *vitro* assay model.

MATERIALS AND METHODS

Acquisition of the experimental shrub

Fresh entire plant of *Anisomeles indica(Linn)* was gathered from in and around Dindugal, Tamil Nadu and authenticated by Dr. P. Jayaraman, taxonomist and Director of the Plant Anatomy Research Centre in Chennai. A voucher shrub sample has been stored in the institution for ready review.

Plant extracts' preparation: Extraction procedure of (Do *et al.,* 2014) was adopted with slight variations .The whole plant of *Anisomeles indica* was washed, left to shade dry and then crushed into granular particles in a mechanical processor and then defatted employing n-hexane as the solvent. The extractions were carried out by soaking 5 g of defatted residue in three different solvents namely distilled water, methanol and ethyl acetate at a sample to solvent ratio of 1:10(weight/volume).Rapid stirring of the mixture for 15-20 minutes in a vortex mixer, followed by centrifugation (13,000g,37^0C)yielded supernatants, which were then concentrated using rotary evaporator (45^0 C) .The concentrated samples thus obtained are referred to as Me OH-AI , EtAc-AI and Aq-AI .They were freeze dried and stored at 4^0C until use.

Qualitative analysis

Phytochemical constituents present in Me OH-AI, EtAc- AI and Aq-AI were analyzed as per the standard protocols (Harborne 1984; Sofowora 1993)

Quantitative analysis

The freeze-dried extract (1 g) obtained from each solvent was dissolved in absolute ethanol (1:10, w/v) and subsequently used for flavonoid, alkaloid and phenol quantification.

Estimation of total flavonoid content: A revised version of (Zhishen *et al.,* 1999) was used to quantify the flavonoids. In a volumetric flask of 10 ml capacity, added 0.1ml of test extracts (10mg/ml), and then distilled water to make up the remaining volume to 5ml. To above mix, addition of 0.3ml of 5% sodium nitrite was done and vortexed it. Set aside the contents at 37^0C for 5 minutes. Additions of 0.3ml of 10% Aluminium chloride reagent and 2 ml of 1M sodium hydroxide to the reaction mix was done, after which the contents of the flask were diluted to 10ml with distilled water. Standard quercetin solution (Concentration range 25-400 µg/mL) was exactly subjected to a similar treatment as the tests. The contents were well mixed. In a UV-spectrophotometer, absorbance of both test and reference solutions at 510 nm was measured versus a reagent blank. The findings were expressed as milligrams of quercetin equivalent (QE)/ gram of extract. The equation for linear regression derived from the standard graph was used to quantify the total flavonoid content of the sample. Total flavonoid content was determined by applying equation 01:

$$\text{Total flavonoid content} = C \times V / m \qquad \text{Eq. 01}$$

Here V denotes the volume of the extract sample (ml), C is the concentration (mg/ml), and m is the weight (g) of the sample extract.

*Estimation of total alkaloid content***:** Bromocresol green reaction with the alkaloid results in the creation of a yellow-colored compound, which forms the basis for this measurement method (Ajanal *et al.,* 2012). A standard solution of atropine was made by dissolving 1 mg of atropine (pure) in 10 mL of distilled water. Aliquots (concentration 25 to 400 µg/ml) from this solution were accurately measured and transferred to various separatory funnels. Portion of EtAc –AI, Me OH –AI and Aq- AI fractions whose solvents had been evaporated, were dissolved perfectly in 2 N hydrochloric acid and filtered. 1mL of each of the three filtrates was then extracted to different separatory funnels and rinsed thrice with 10mL of chloroform. The reaction of

the mixture was brought to neutral with 0.1N sodium hydroxide after which both standard and test extracts were treated with 5ml phosphate buffer (pH4.7) and 5ml of bromocresol green reagent. After final vigorous shaking with 1,2,3 and 4ml of chloroform, extracts were then recovered in separate 10ml volumetric flask and adjusted using chloroform reagent. With the aid of a UV-Spectrophotometer, absorbance was evaluated at 470 nm, in comparison to a blank produced similarly, but without Atropine. From the atropine standard graph calibration, alkaloid content was quantified and expressed as equivalents of atropine (milligrams)/gram of extract.

Estimation of total phenol content: Folin-Ciocalteau reagent method was used to assess total phenol concentration of the Aq-AI, Me OH –AI and Et Ac –AI fractions (Parvin *et al.,* 2015). In this method To each(1ml) of the Aq-AI, Me OH –AI and EtAc –AI fractions, Folin-Ciocalteau reagent (5 ml, 10% v/v) and sodium carbonate saturated solution (2ml) were added. Absorbance of the samples, versus a reagent blank was measured in a spectrophotometer (765nm), after the reaction contents were allowed to rest at 37^0C for 30 minutes. Standard Gallic acid solution (25µg/ml- 400 µg/ml) prepared from stock of 1mg/ml, were also treated similarly. The gallic acid standard graph was calibrated to evaluate the phenol contents in the fractions and the findings were expressed as equivalents of Gallic acid (milligrams)/gram of extract. Total phenol content was determined by employing the equation (02):

$$\text{Total phenol content} = C \times V / m \qquad \text{Eq. 02}$$

Here V denotes the volume of sample (ml), C is the concentration (mg/ml) and m is the weight (g) of sample extract.

***In Vitro* Anti-Inflammatory Activity**

Inhibition of albumin denaturation: A standard 0.1% solution of the positive control drug was prepared by precisely weighing 0.2g of Ibuprofen (NSAID) powder and dissolving it in 200 ml of double distilled water. Contents were then vortexed to yield a uniform solution (Handa *et al.,* 2008). Serial dilutions of Aq-AI, Me OH –AI and Et Ac –AI extracts was performed using double distilled water, to give final concentrations of 25 to 1000 µg/ml. Double distilled water alone was used as negative reference. Experiment was performed using a modified version of the technique described by Kumari *et al.,* (2015). Into a series of reaction vessels, added 0.2 ml of fresh albumin isolated from a hen's egg followed by 2.8 ml of phosphate-buffered saline (pH 6.4).Then aliquots of 2 ml from each of the serially diluted Aq-AI, Me-OH-AI, and Et Ac –AI fractions were also gently added into the above reaction mix , so as to yield a final volume of 5.0 ml. The mixture was maintained at 37 °C (15 minutes) in a water bath before being heated to 70^0 C (5 minutes). The temperature of the reaction mix was brought to 37^0C and the turbidity measured in a spectrophotometer at 660nm versus a pure blank. Ibuprofen at equivalent doses to test extracts was treated similarly for absorbance determination. The % inhibition of albumin denaturation was computed as follows:

$$\text{Percentage inhibition} = (\text{Absorbance}_{Control} - \text{Absorbance}_{Sample}) \text{ X } 100 / \text{Absorbance}_{control}$$

Statistical Analysis

All estimations were conducted thrice and the findings were presented as mean values with standard deviations (±SD). Microsoft Excel 2013(Roselle, IL, USA) was adopted for the graphic as well as statistical analytics .Dose response curve analytic tool in the Graph Pad Prism software version 8.0 was employed to assess the 50% inhibitory concentration of all samples.

RESULTS AND DISCUSSION

Results

Table 1: Qualitative Phytochemical screening of various solvent extracts of *Anisomeles indica* **(whole plant). Absent -, Low +, Medium ++, High +++. Et ac -AI- Ethyl acetate extract of *Anisomeles indica*, Aq-AI - Aqueous extract of *Anisomeles indica,* Me OH -AI Methanol extract of *Anisomeles indica.***

Constituents	Et ac-AI	Me OH -AI	Aq- AI
Alkaloids	+	++	+++
Anthraquinone	-	-	-
Flavonoids	+	+++	++
Glycosides	-	+	-
Phytosterol	-	-	-
Phenols	++	+++	++
Sterols	+	-	-
Proteins	+	+	+
Terpenoids	+	-	-
Triterpenes	+	-	+
Tannins	-	-	-

Table 2: Alkaloid contents in the *Anisomeles indica* extracts expressed as the equivalent of atropine (AE) per gram of extract. Each of these values represents the standard deviation of the three data points.

Extract	mg AE/gram of extract
Et ac-AI	39.12±0.043
Me OH –AI	77.09 ±0.012
Aq-AI	102.61±0.002

Table 3: Flavonoid contents in the extracts of *Anisomeles indica* expressed as the equivalent of quercetin (QE) per gram of extract. Each value is the average of three analyses ± standard deviation.

Extract	mg of QE/gram extract
Et Ac-AI	76.08±0.032
Aq-AI	109 .92 ± 0.015[1]
Me OH -AI	133.55±0.011

Table 4: Phenol contents in the extracts of *Anisomeles indica* expressed as the equivalent of gallic acid (GAE) per gram of extract. Each value is the average of three analyses ± standard deviation.

Extract	mg of GAE/gram extract
Et ac-AI	80.53±0.014
Aq-AI	124.56± 0.012
Me OH -AI	148.50±0.023

Table 5: Effect of whole plant extracts of *A .indica* and reference drug on albumin denaturation assay for anti-inflammatory activity. Results are shown as mean ± SD. (n = 3).

Concentration (µg/ml)	Percentage of inhibition			
	Et ac - AI	**Aq -AI**	**Me OH AI**	**Ibuprofen**
25	20.9 ± 0.023	45.01±0.031	58.34±0.041	59.15± 025
50	25.1± 0.043	50.75±0.030	64.11± 0.021	66.03±0.064
100	31.15 ± 0.050	54.01±0.047	70.03 ±0 .024	71.61 ±0.013
250	36.02±0.021	61.43 ± 0.02	77.18± 0.061	79.01 ±0.08
500	39.73±0.09	66.47 ±0.06	82.74 ±0.072	84.91±0.078
1000	42.96±0.12	71.09± 0.075	85.12± 0.078	87.45± 0.01

Table 6: 50% inhibitory concentration (IC50) values of inhibition of albumin denaturation activity in different *Anisomeles indica* whole plant extracts and reference drug. Et ac -AI- Ethyl acetate extract of *Anisomeles indica*, Aq-AI - Aqueous extract of *Anisomeles indica,* Me OH -AI Methanol extract of *Anisomeles indica*.

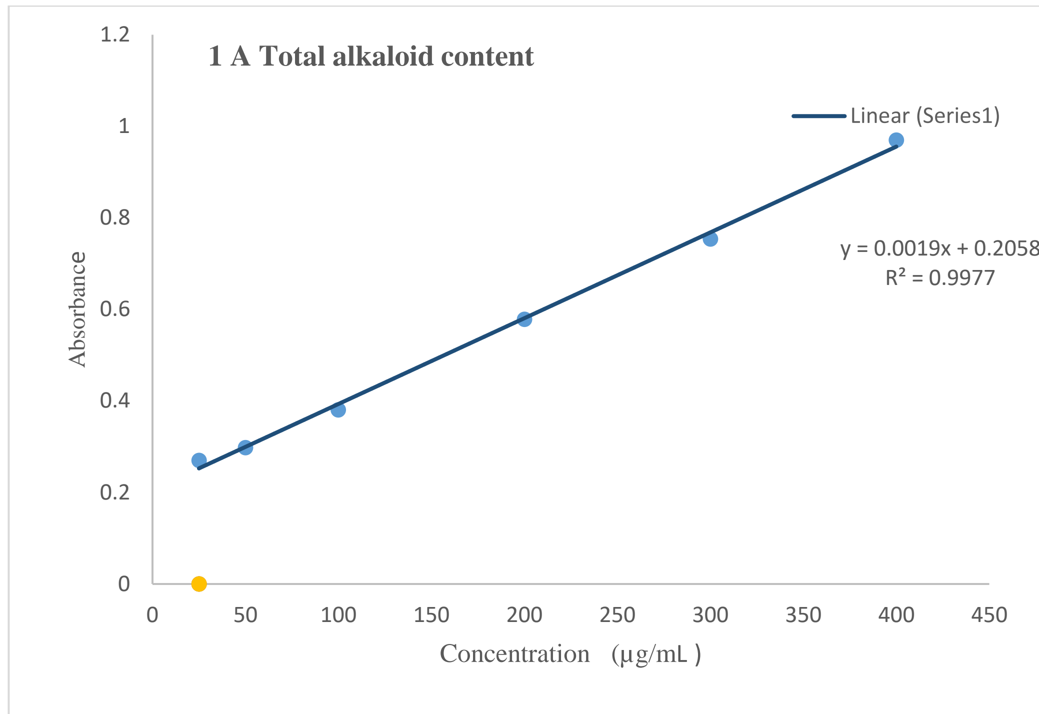
Sample	IC50 value µg/ml	Correlation coefficient (r^2) value
Et ac -AI	2782.84	0.9907
Aq-AI	49.904	0.9971
Me OH-AI	7.461	0.99
Ibuprofen	6.785	0.9901

From the current investigation, it may be concluded that the relative concentration in the three extracts of *Anisomeles indica* (L.) investigated were in the order - Phenols > Flavonoids > Alkaloids>Proteins > Triterpenes>Terpenoids >Glycosides in the methanol extract of *Anisomeles indica*. As reported previously, presence of secondary metabolites in plants contribute to its pharmacological activities and its application in traditional medicine(Ulhe and Narkhede, 2013).Methanol extract showed a predominance of phenols, flavonoids followed by alkaloids, whereas in the ethyl acetate and aqueous fractions, presence of proteins, terpenoids and triterpenes were also indicated in addition. (Table 1) Phyto constituents such as glycosides, triterpenes and sterols were less while phytosterols, tannins and anthraquinones were totally absent.

Total alkaloid content: The absorbance data of standard Atropine at various concentrations was used to plot a calibration curve (Fig.1A). Based on the straight line equation from graph (y=0.0019x+0.2058; r^2 value - 0.9977), alkaloid content in samples were calibrated. Elevated levels of alkaloid (102.61±0.002) was observed in the aqueous extract (Fig.1B; Table2). Alkaloid content was expressed as the equivalent of atropine mg/g of extract.

Total flavonoid content: in the samples was evaluated with the help of quercetin standard calibration curve (Fig.2A) (y = 0.0021x + 0.1497, r^2 =0.9907). A variation in the flavonoid concentration of the whole plant extracts of *Anisomeles indica* was recorded with methanol extract demonstrating the highest value of (133.55±0.011 mg of QE/gm. of extract, accompanied by aqueous extract (109.92± 0.015) (Table3; Fig. 2B).

Total phenol content: Results were calibrated employing the absorbance data from the (gallic acid) straight line graph, at different concentrations (0–400 µg/ml) (Fig.3A). The r^2value (0.9942) for the equation y=0.0017x+0.1333 is indicative of the goodness of fit. Total phenol was expressed as equivalent of gallic acid mg/gram of extract. A leading phenolic content of 148.50±0.023 and 124.56± 0.012 mg of equivalent of gallic acid per gram of crude extract was observed in the methanol and aqueous extracts respectively (Table 4; Fig.3B).



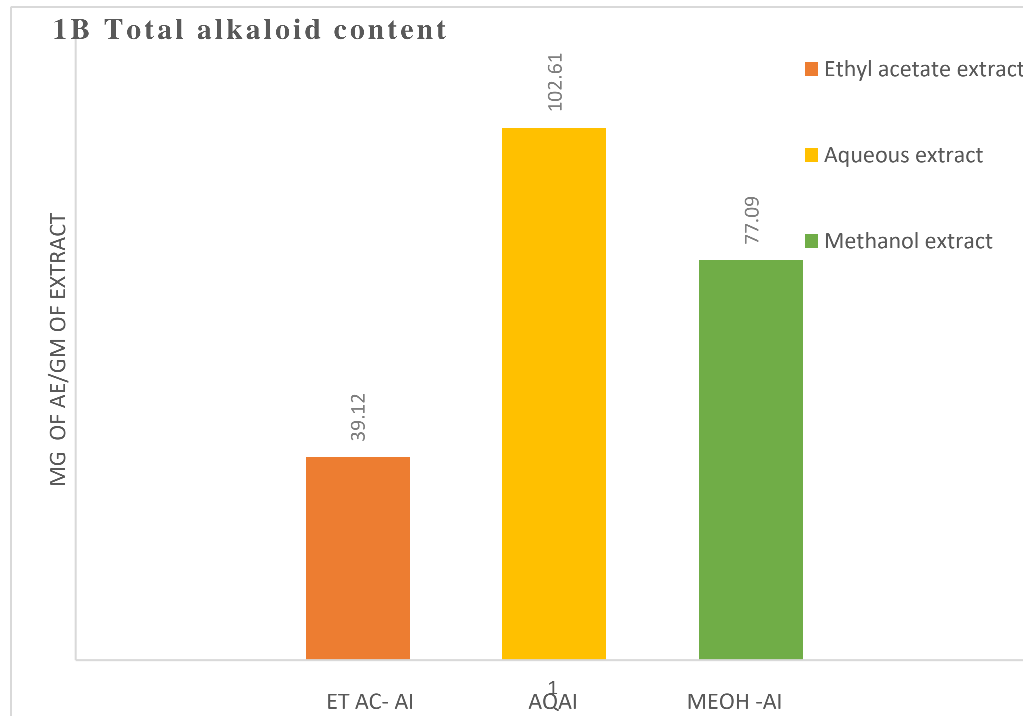


Figure 1: A- Standard calibration graph for total alkaloid content in the whole plant extracts of *Anisomeles indica;* B- Total Alkaloid content in extracts of *Anisomeles indica*. Values are represented as mg of AE/g of extract. Each value in the graph is represented as mean ± SD (n = 3). ETAC -AI- ethyl acetate extract of *Anisomeles indica*, Aq-AI - aqueous extract of *Anisomeles indica,* MEOH -AI methanol extract of *A.indica*

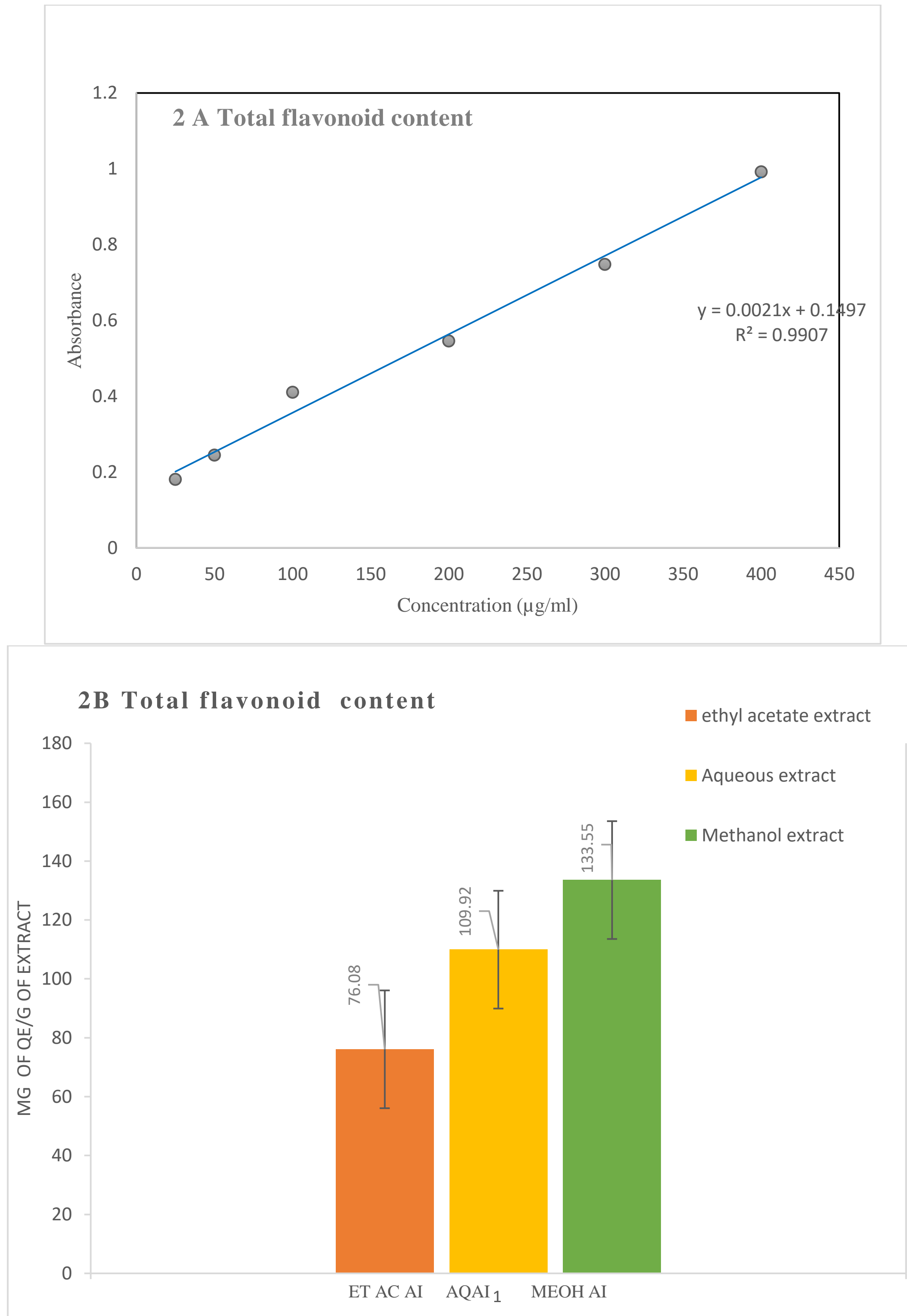


Figure 2: A- Standard calibration graph for total flavonoid content in the whole plant extracts of *Anisomeles indica;* B- Total flavonoid content in extracts of *Anisomeles indica*. Values are represented as mg of QE/g of extract. Each value in the graph is represented as mean ± SD (n = 3).

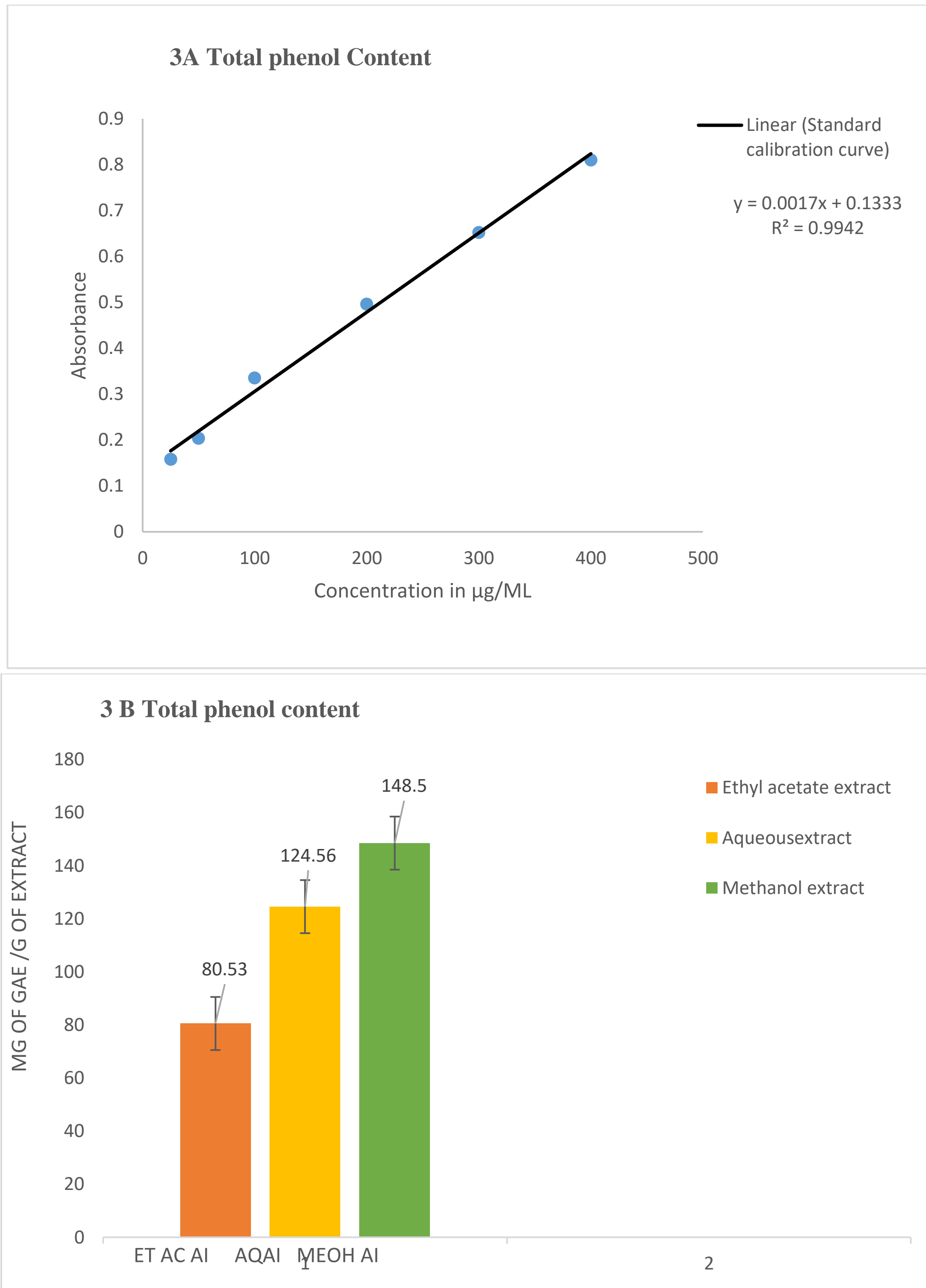


Figure 3: A- Standard calibration graph for total phenol content in the whole plant extracts of *Anisomeles indica;* B- Total phenol content in extracts of *Anisomeles indica*. Values are represented as mg of GAE/g of extract. Each value in the graph is represented as mean ± SD (n = 3).

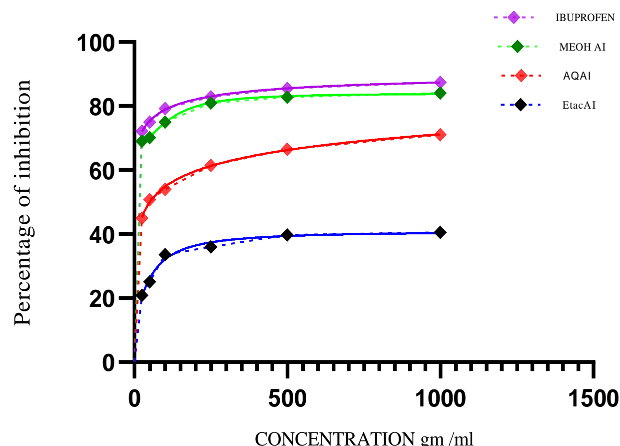


Figure 4: IC 50 value of *Anisomeles indica* extracts and standard reference drug for inhibition of albumin denaturation activity.

*Inhibition of albumin denaturation***:** This research evaluated**,** the *in vitro* anti-inflammatory efficiency of methanol, aqueous and ethyl acetate extracts of *Anisomeles indica* was measured for inhibitory activity against albumin denaturation. Methanol extract (Me OH-AI) was identified as the most highly effective inhibitor of albumin denaturation (85.12± 0.078), followed by aqueous extract (71.09± 0.075) at the concentrations of 1000µg (Table 5).This was further confirmed by comparing the IC50 values of methanol extract which was 7.461 μg/ml at correlation coefficient value (r^2) of 0.99 and that of the Aq-AI extract, 49.904 μg/ml at correlation coefficient value of 0.9971(Table 6). IC_{50} value of 2782.84 µg/ml denoting the least capability to prevent albumin gelation was demonstrated by the ethyl acetate fraction (Fig.8). According to the current investigation, the three solvent fractions of *A. indica* and also ibuprofen (reference NSAID) exhibited protein protecting capabilities in a concentration-dependent manner over the concentration range of 25µg /ml to1000 µg /ml. Results (Table 5) demonstrated elevated rates of more than 50% inhibition throughout the selected concentration levels of 25 to 1000 μg/mL for the NSAID. Ibuprofen exhibited the highest inhibition percentage of 87.45. In addition, IC 50 value of methanol extract of *A. indica* (7.461 μg/ml) is equivalent to NSAID ibuprofen synthetic drug (6.785 μg/ml), routinely used for combating inflammation (Fig .4). Consequently, this study has offered additional proof of the plant's prospective anti-inflammatory capability.

Discussion

The inflammatory process is a compensatory reaction mechanism of biological membranes to stressors such as physical injury, widespread infection, toxic chemical irritants, and the production of inflammatory mediators from injured cell structures (Sangeetha &Vidhya, 2016; Leelaprakash & Mohan Dass, 2010). These agents tend to disrupt the bonds that stabilize the plasma membrane's native protein structure, resulting in increased protein denaturation, cellular membrane disintegration, elevated vascular permeability (Leelaprakash and Mohan Dass, 2010)) and tissue injury (Ingle and Patel, 2011; Opie, 1962). Inflammation symptoms include swelling, redness, pain, and altered physiological processes. Persistent inflammation has been related to the onset of several number of diseases, including arthritis, cancer, and stroke. Inflammatory activity, however, can be lowered by blocking protein denaturation (Osman *et al.,* 2016). Due to their ability to prevent protein denaturation, NSAIDs are among the commonly prescribed medicines recommended by the physicians for the management of inflammatory conditions (Osman *et al.,* 2016). Ibuprofen is one such anti-inflammatory medicine that is routinely recommended by physicians; nonetheless, there has been a documented risk of major adverse effects such as bleeding, ulcers with long-term usage. (Langman *et al.,* 1994). Since the bioactive compounds of plant origin are safer and have fewer negative effects than conventional therapies (Sangeetha and Vidhya, 2016), this study focusses on the ability of *Anisomeles indica* extracts to prevent protein denaturation *in vitro* by employing the albumin denaturation assay. Findings revealed that methanol extract

of *Anisomeles indica* was potent in decreasing albumin denaturation. Highest rates of inhibition of 85.12% was recorded at 1000 µg/ml and 82.74 % at 500µg/ml. At the concentration of 1000 µg/ml, Ibuprofen demonstrated a maximal inhibitory effect of 87.45%.on protein denaturation. High protein protective capabilities and lower IC50 values similar to that of Ibuprofen, was seen in the methanol fraction which may be ascribed to its comparatively higher concentrations of flavonoids and phenolic compounds (Hoang *et al.,* 2015) than the aqueous or ethyl acetate extracts .This study reinforces that methanol extract of *Anisomeles indica* is both an effective and potential storehouse of anti-inflammatory compounds, which needs to be explored and maximally utilized in therapy and also confirming its conventional application to a variety of inflammatory disorders.

Conclusion

According to the findings of this study, Methanol is an ideal vehicle for extracting the phytoconstituents with protein-protective properties, present in *Anisomeles indica.* The extract's anti-inflammatory properties are likely due to its high phenol and flavonoid content. Additional research is needed to optimally develop the bioactive principles of *Anisomeles indica* as therapeutic formulations in inflammation therapy.

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