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QUALITATIVE AND QUANTITATIVE ANALYSIS OF PHYTOCHEMICALS AND EVALUATION OF *IN-VITRO* ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF THREE SELECTED AYURVEDIC MEDICINAL PLANTS USED IN RHEUMATOID ARTHRITIS MEDICATIONS

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Keywords:

Rheumatoid arthritis, antioxidant activity, anti-inflammatory activity, *Gmelina arborea*, *Boehmeria nivea*, *Oroxylum indicum*

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ABSTRACT: This research was focused on the qualitative and quantitative analysis of phytochemicals and evaluation of *in-vitro* antioxidant and anti-inflammatory activities of three commonly used plant parts in rheumatoid arthritis medications in Ayurveda, namely, the root of *Boehmeria nivea*, the barks of *Gmelina arborea* and *Oroxylum indicum*, using standard methods. Qualitative analysis of each methanol extract confirmed the presence of alkaloids, flavonoids, condensed tannins, polyphenols, triterpenes, sterols, and saponins in varying quantities. The highest total alkaloid content of 108.81 ± 0.54 mg caffeine equivalent per 100 g of the dry weight of plant materials (DW) and the highest total flavonoid content of 268.94 ± 12.62 mg catechin equivalent per 100 g DW were exhibited by the bark extracts of *Gmelina arborea* and *Oroxylum indicum*, respectively. The highest total condensed tannin content of 529.34 ± 30.51 mg catechin equivalent per 100 g DW and the highest total phenolic content of 640.34 ± 64.58 mg gallic acid equivalent per 100 g DW were demonstrated by the root extract of *Boehmeria nivea*. The highest antioxidant activity with IC_{50} value of 30.82 ± 2.49 mg/L and the highest anti-inflammatory activity with IC_{50} value of 119.44 ± 0.25 µg/mL were demonstrated by the bark extract of *Gmelina arborea*. The correlation of the anti-inflammatory activity against the total flavonoid content demonstrated a strong positive correlation with a Pearson's correlation coefficient of 0.9568. Plant parts analyzed in this study are enriched with natural antioxidants and anti-inflammatory agents.

INTRODUCTION: Rheumatoid arthritis (RA) is a progressive, inflammatory and chronic autoimmune disorder that causes long-term joint pain, stiffness, swelling, warmth, and redness around the affected joints [1, 2].

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Genetic, environmental, hormonal, and lifestyle factors are identified as potential causations of RA [3]. The treatments for RA in Western medicine use several classes of drugs, including disease-modifying anti-rheumatic drugs, non-steroidal anti-inflammatory drugs (NSAID), analgesics, and corticosteroids which temporarily suppress the symptoms of RA while causing several side effects [2, 4, 5].

However, treatments for RA in ayurvedic medicine provide a permanent cure without causing any side effects for the patient [6].

Several herbal preparations, including '*Dhanwantharam thaila*', '*Sahacharadi thaila*' and' *Narayana thaila*', which are used to treat RA patients in ayurvedic medicinal system, the root of *Boehmeria nivea*, barks of *Gmelina arborea* and *Oroxylum indicum* have been frequently used [7, 8].

Boehmeria nivea (Urticaceae), commonly known as 'ramie' in English, is an upright deciduous, monoecious sub-shrub or shrub that typically grows eight to ten inches tall and widely distributed in South Korea, India, China, and Japan [9, 10]. The edible root of *Boehmeria nivea* has a sweet taste and demonstrates several biological properties, including antioxidant, antipyretic, demulcent, diuretic and hepatoprotective properties [9]. *Gmelina arborea* (Verbenaceae), commonly known as 'beech wood' in English is a fast-growing deciduous tree species found in the Southern and South-eastern Asia [11]. The immature bark of *Gmelina arborea* appears in light gray or gray-yellow and becomes brown and rough upon maturation. Anti-diarrheal, antidiabetic, anthelmintic, anti-epileptic, antimicrobial, diuretic, and hepatoprotective properties of the bark of *Gmelina arborea* are extensively used in traditional medicine [12]. *Oroxylum indicum* (Bignoniaceae), commonly known as 'midnight horror' is a medium-sized deciduous, flowering tree that is native to the Indian subcontinent and distributed in the Himalayan foothills, Southern China, Indochina, and Malaysia [13]. The bark of *Oroxylum indicum* is found to possess antidiabetic, antimicrobial, and hepatoprotective properties [13, 14].

Although certain qualitative and quantitative phyto-chemical analysis and the evaluation of some of the biological properties of above mentioned three selected plant parts which were grown in various regions of the globe have been reported, a systematic study on the plant parts of above mentioned three selected medicinal plants grown in Sri Lanka is still lacking. Therefore, this research is focused on a systematic study on qualitative and quantitative analysis of phytochemicals, evaluation of *in-vitro* antioxidant and anti-inflammatory properties along with the correlation analysis of methanol extracts of three extensively used plant parts in RA medications, namely, the root of *Boehmeria nivea*, barks of *Gmelina arborea*, and *Oroxylum indicum* which were grown in Sri Lanka.

MATERIALS AND METHODS:

Chemicals: All chemicals, reagents, and solvents were of analytical grade and used as received from chemical suppliers without any purification unless otherwise mentioned.

Instruments: An electrical thermostatic water bath (Nickel Electro Ltd, England), heating mantle (European Economic Community, 230 V, 50/60 Hz, 130 W), centrifuge machine (HERMLE Labortechnik GmbH, Z 206 A), analytical balance (Precisa Instruments Ltd, max: 200 g min: 0.0001 g), pH meter (Eutech Instruments, pH 700), UV-visible spectrophotometer (LabomedInc, UVD-2960), blender (SINGER PLC, Sri Lanka, KA-PB-426), and rotary evaporator (Qualitron (Pvt) Ltd, laborota 4000) were used.

Collection of Plant Samples: The root of *Boehmeria nivea* and bark of *Gmelina arborea* were collected from the geographical coordinates of 6°49'35.9"N 80°09'29.7"E and 6°49'35.9"N 80°09'29.5"E, respectively, in Handapangoda area Western Province, Sri Lanka. The bark of *Oroxylum indicum* was collected from the geographical coordinates of 6°51'41.7"N 79°56'33. 2"E in Palanwaththa area Western Province, Sri Lanka. All of the above mentioned plant parts were collected during the month of February, 2019. The identification and authentication of plant species were carried out by Mrs. Pushpa Jeewandara, Research Officer of the Division of Pharmaceutical Botany in Bandaranayaka Memorial Ayurvedic Research Institute, Nawinna, Maharagama, Sri Lanka. Sample voucher specimens of *Gmelina arborea*, *Oroxylum indicum*, and *Boehmeria nivea* with accession numbers 2051, 2052, and 2053, respectively, were deposited in the aforementioned institute.

Preparation of the Extracts: The methanol extracts of above-mentioned plant materials were prepared separately using modified literature procedures as follows [15–17]. Cleaned plant materials were washed with distilled water and dried under the shade over 6 h per day for 10 days, and ground into a uniform powder using a blender. Then the weight of 100 g of powdered material was subjected to Soxhlet extraction using methanol over 6 h. The concentrated extract was stored at 4 °C until further use.

Determination of Percentage Extractable Yield: The methanol extract of 10.000 g of plant material (W_1) was prepared as explained in the 'preparation of the extracts' section above. The resultant methanol extract was evaporated to dryness using a rotary evaporator until the weight of the dried extract became constant. The final weight of the concentrated plant extract was (W_2) recorded. The extractable yield was calculated using the equation given below.

$$\% \text{ Extractable yield} = W_2/W_1 \times 100\%$$

Qualitative Analysis of Phytochemicals: The phytochemicals were screened using modified literature procedures [18, 19]. Alkaloids were screened using the Wagner's and Mayer's tests, while flavonoids were screened using the cyanidin test. Tannins and polyphenols were screened using the gelatine test and $FeCl_3$ test, respectively. The presence or absence of sterols and triterpenes were determined using the Liebermann-Burchard and Salkowski tests, respectively. Saponins were screened using the froth test. The observations of each test were recorded with respect to the control sample.

Quantitative Analysis of Phytochemicals: The total alkaloid content (TAC), total flavonoid content (TFC), total condensed tannin content (TCTC) and total phenolic content (TPC) of methanol extracts of the root of *Boehmeria nivea* and the barks of *Gmelina arborea* and *Oroxylum indicu*m were determined by employing the standard spectrophotometric methods, bromocresol green, aluminium chloride, butanol–hydrochloric acid–iron and Folin–Ciocalteu methods, respectively [18–22]. Caffeine was used as a reference compound for the TAC determination. The TFC and TCTC were determined using catechin as the reference compound. Gallic acid was employed as the reference compound for the TPC determination. Absorbance measurements for the determination of TAC, TFC, TCTC, and TPC were recorded at 470 nm, 510 nm, 550 nm, and 765 nm, respectively. Each absorbance measurement was triplicated and the mean value was taken.

Determination of *In-vitro* Antioxidant Activity: *In-vitro* antioxidant activity was determined using 2, 2-diphenyl-1-picrylhydrazyl (DDPH) assay [22, 23]. Ascorbic acid was used as the reference compound. The absorbance of each solution which was measured at 517 nm wavelength was triplicated. The percentage DPPH radical scavenging activity of the concentration series of each plant material and the standards was calculated using the equation given below.

$$\% \text{ DPPH radical scavenging activity} = [(A - A')/A] \times 100\%$$

Where A' is the absorbance of the plant extract or the reference compound and A is the absorbance of the control without the plant extract or the reference compound.

Determination of *In-vitro* Anti-inflammatory Activity: *In-vitro* anti-inflammatory activity was evaluated using proteinase inhibitory assay by using diclofenac sodium as the reference compound [24]. The absorbance of each solution was measured at 210 nm wavelength and triplicated. The percentage of proteinase inhibitory activity was calculated for each solution in the concentration series of standards and samples using the equation given below.

$$\% \text{ Proteinase inhibitory activity} = [(A - A')/A] \times 100\%$$

Where A' is the absorbance of the plant extract or the reference compound and A is the absorbance value of the control without the plant extract or the reference compound.

Correlation Study and Statistical Analysis: Correlations between the anti-inflammatory activity of methanol extract of the root of *Boehmeria nivea*, barks of *Gmelina arborea*, and *Oroxylum indicum* with their corresponding TAC, TFC, TCTC, and TPC were investigated separately by plotting the anti-inflammatory activity *vs.* TAC or TFC or TCTC or TPC. The Pearson's correlation coefficient (R) and linear regression (R^2) value of each plot were calculated using Origin 9.0 software.

RESULTS AND DISCUSSION: The World Health Organization reported that 80% of the world population depend on traditional medicine for their primary health care needs [25]. The majority of treatment methods in traditional medicine utilizes biologically active herbal extracts and their phyto-constituents which inherit a vast range of medicinal and pharmacological properties against numerous chronic as well as acute diseases and disorders [26]. In this study, three selected plant parts which are

used in the RA medications of ayurvedic medicine were subjected to qualitative and quantitative analysis of phytochemicals followed by the evaluation of their *in-vitro* antioxidant and anti-inflammatory activities.

Determination of Percentage Extractable Yield: The percentage extractable yield of each selected plant material was calculated to determine % w/w of plant secondary metabolites in each plant material **Table 1**.

Among the three methanol extracts analyzed, the bark of *Gmelina arborea* demonstrated the highest percentage extractable yield of 6.84 ± 0.12% w/w, whereas the bark of *Oroxylum indicum* exhibited the lowest percentage extractable yield of 1.66 ± 0.11% w/w.

TABLE 1: THE PERCENTAGE EXTRACTABLE YIELDS OF METHANOL EXTRACTS OF THREE SELECTED PLANT PARTS

Plant material	Percentage extractable yield (% w/w)
Root of *Boehmeria nivea*	6.62 ± 0.53
Bark of *Gmelina arborea*	6.84 ± 0.12
Bark of *Oroxylum indicum*	1.66 ± 0.11

Values were expressed as mean ± standard deviation (SD) of three parallel measurements

Qualitative Analysis of Phytochemicals: Phyto-chemical screening of selected plant materials was carried out to determine the availability of different classes of natural products in each plant extract **Table 2**. In the qualitative analysis of phyto-chemicals, the formation of a yellow precipitate for the Mayer's test and a reddish-brown precipitate for the Wagner's test indicated the presence of alkaloids. A comparative analysis on the resultant precipitate confirmed that the bark of *Gmelina arborea* constitutes the highest amount of alkaloids. The formation of a red color solution for the cyanidin test confirmed the presence of flavonoids. The intensity of the red color solution indicated that the bark of *Oroxylum indicum* extract consists of comparatively high amount of flavonoids while the root of *Boehmeria nivea* and the bark of *Gmelina arborea* extracts exhibit a moderate amount of flavonoids.

The formation of white color precipitates for the gelatin test and deep-violet color solution for the $FeCl_3$ test evidenced the presence of tannins and polyphenols, respectively. The blue-green color solution formation for the Liebermann-Burchard test and the cherry-red color interface formation between organic and aqueous layers for the Salkowski test with varying intensities confirmed the presence of sterols and triterpenes, respectively in different amounts. The methanol extracts of the bark of *Gmelina arborea* and root of *Boehmeria nivea* exhibit the highest and lowest amount of sterols and triterpenes, respectively. The persistent honeycomb froth formed above the surface of the solution for the froth test indicated the presence of saponins. The bark of *Gmelina arborea* demonstrates a comparatively high amount of saponins.

TABLE 2: QUALITATIVE ANALYSIS OF PHYTOCHEMICALS IN METHANOL EXTRACTS OF THREE SELECTED PLANT PARTS

Plant part	Alkaloids		Flavonoids	Tannins and Polyphenols		Sterols and Triterpenes		Saponins
	Mayer's test	Wagner's test	Cyanidin test	Gelatin test	$FeCl_3$ test	Liebermann-Burchard test	Salkowski test	Froth test
Root of *Boehmeria nivea*	++	++	++	++	++	+	+	+
Bark of *Gmelina arborea*	+++	+++	++	++	++	+++	+++	+++
Bark of *Oroxylum indicum*	+	+	+++	++	++	++	++	++

Where; +, slight amounts; ++, moderate amounts; +++, large amounts, and – not detectable, of the corresponding phytochemical(s)

Quantitative Analysis of Phytochemicals:

Total Alkaloid Content: The TAC of all samples **Table 3** were expressed as milligrams of caffeine equivalent per 100g dry weight of plant material (mg CFE/100 g DW) based on the calibration curve of caffeine which has the R^2 of 0.9975; y = 0.0049 x + 0.0473. The quantitative analysis of alkaloids evidenced that the bark extracts of *Gmelina arborea* and *Oroxylum indicum* demonstrated the highest TAC of 108.81 ± 0.54 mg CFE/100 g DW and the lowest TAC of 28.94 ± 2.34 mg CFE/100 g DW, respectively.

Total Flavonoid Content: The TFC of three selected plant materials **Table 3** were expressed as milligrams of catechin equivalent per 100 g dry weight of plant material (mg CTE/100 g DW) based on the calibration plot of catechin which has the R^2 of 0.9935; y = 0.0007 x + 0.0832. In the quantification of flavonoids the bark extract of *Oroxylum indicum* exhibited the highest TFC of 268.94 ± 12.62 mg CTE/100 g DW whereas the root extract of *Boehmeria nivea* and bark extract of *Gmelina arborea* exhibited the TFC of 124.76 ± 9.64 mg CTE/100 g DW and 148.11 ± 31.30 mg CTE/100 g DW, respectively. The TFC of cold methanol extract of the root of *Boehmeria nivea* grown in Guangzhou, China, was reported as 636.9 ± 44.2 mg CTE/100 g DW [27]. The TFC of the root of *Boehmeria nivea* reported by Wang *et al.* [27] is incomparable with that of this reporting study because of the differences in the extraction temperature. Attanayake *et al.*, [28] described that the TFC of aqueous extract of bark of *Gmelina arborea* grown in the Southern part of Sri Lanka as 1.77 ± 0.1 µg of quercetin equivalent (QE)/g DW. The differences of the standard compound and the solvent restrained the comparison of TFC of bark extract of *Gmelina arborea* of this reporting study with the study of Attanayake *et al.* [28]. Furthermore, the TFC of 2.075 mg CTE/g DW for methanol extract of bark of *Oroxylum indicum* grown in Andra Pradesh, India which was lower than that of this reporting study [29]. Moreover, Singh *et al.* [30] evaluated the TFC of methanol extract of bark of *Oroxylum indicum* grown in Manipur, India as 346.6 ± 15.2 mg QE/g of DW. Although the TFC of methanol extract of bark of *Oroxylum indicum* grown in Manipur, India was reported as 346.6 ± 15.2 mg QE/g of DW, the use of different standard compounds limited the comparison of it with this study [30].

TABLE 3: QUANTITATIVE ANALYSIS OF SECONDARY METABOLITES, ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF METHANOL EXTRACTS OF THREE SELECTED PLANT MATERIALS

Plant Material	Total Alkaloid Content (mg CFE/ 100 g DW)	Total Flavonoid Content (mg CTE/ 100 g DW)	Total Condensed Tannin Content (mg CTE/ 100 g DW)	Total Phenolic Content (mg GAE/ 100 g DW)	Antioxidant Activity - IC_{50} (mg/L)	Anti-inflammatory Activity - IC_{50} (µg/mL)
Root of *Boehmeria nivea*	60.70 ± 2.36	124.76 ± 9.64	529.34 ± 30.51	640.34 ± 64.58	33.13 ± 2.50	134.75 ± 0.36
Bark of *Gmelina arborea*	108.81 ± 0.54	148.11 ± 31.30	445.31 ± 8.00	583.42 ± 30.43	30.82 ± 2.49	119.44 ± 0.25
Bark of *Oroxylum indicum*	28.94 ± 2.34	268.94 ± 12.62	512.82 ± 53.33	591.60 ± 40.99	36.43 ± 2.56	210.06 ± 2.5

Where; mg CFE/100 g DW – milligrams of caffeine equivalent per 100 g of dry weight, mg CTE/100 g DW – milligrams of catechin equivalent per 100 g of dry weight, mg GAE/100 g DW – milligrams of gallic acid equivalent per 100 g of dry weight, and IC_{50} – 50% inhibitory concentration. Values were expressed as mean ± SD of three parallel measurements.

Total Condensed Tannin Content: The TCTC of the three selected plant materials **Table 3** were expressed as mg CTE/100 g DW based on the calibration curve of catechin which has the R^2 of 0.9740; y = 0.0001 x + 0.031. The quantification of condensed tannins in the root extract of *Boehmeria nivea*, bark extracts of *Gmelina arborea*, and *Oroxylum indicum* resulted in TCTC of 529.34 ± 30.51, 445.31 ± 8.00, and 512.82 ± 53.33 mg CTE/100 g DW, respectively.

Total Phenolic Content: The TPC of selected plant parts **Table 3** was expressed as mg of gallic acid equivalent per 100g dry weight of plant material (mg GAE/100 g DW) based on the calibration curve of gallic acid which has the R^2 of 0.9963; y = 0.0102 x + 0.0026. The studies on TPC evidenced that the highest TPC of 640.34 ± 64.58 mg GAE/100 g DW in the root of *Boehmeria nivea* whereas the TPCs of 591.60 ± 40.99 and 583.42 ± 30.43 mg GAE/100 g DW in barks of *Oroxylum indicum* and *Gmelina arborea*, respectively.

Wang *et al.* [27] reported the TPC of 442.8 ± 9.8 mg GAE/100 g DW for cold methanol extract of the root of *Boehmeria nivea* grown in Guangzhou, China which was lower than the TFC of hot methanol extract of the same plant material used in this study. The differences in geographical location, sample collecting time, and extraction temperature may cause the differences in TFC. Attanayake *et al.* [28] explained that the TPC of aqueous extract of bark of *Gmelina arborea* which grown in the Southern region, Sri Lanka as 13.00 ± 1.10 mg GAE/g of DW. The methanol extract of bark of *Oroxylum indicum* grown in Andra Pradesh, India [29] and Manipur, India [30] exhibited the TPC of 15.5 ± 0.044 and 320.7 ± 34.6 mg GAE/100 g DW, respectively, which were lower than that of the present study.

Determination of *In-vitro* Antioxidant Activity: Natural antioxidants serve as valuable therapeutic agents to reduce the health conditions caused by oxidative stress, including RA, by neutralizing the reactive oxygen species to prevent oxidative damage to cells and tissues [31]. Therefore, the *in-vitro* antioxidant potential of the above mentioned three plant parts used in RA medications in ayurvedic medicine was evaluated.

The antioxidant activities **Table 3** of the standard compound and the plant extracts were expressed as IC_{50} values in mg/L, which is the concentration of the sample where the percentage radical scavenging activity was reduced by half. The IC_{50} values of plant materials were determined using the graph of percentage DPPH radical scavenging activity *vs.* concentration of the standard or sample. The reference compound, ascorbic acid, possesses the antioxidant activity with IC_{50} value of 2.77 ± 2.50 mg/L in methanol. Among the three methanol extracts analyzed, the bark of *Gmelina arborea* exhibited the highest antioxidant activity with IC_{50} value of 30.82 ± 2.49 mg/L while the bark of *Oroxylum indicum* demonstrated the lowest antioxidant activity with IC_{50} value of 36.43 ± 2.56 mg/L.

The *in-vitro* antioxidant activity of aqueous extract of bark of *Gmelina arborea* grown in the Southern region, Sri Lanka was reported as 139.56 ± 4.20 µg/mL [28]. Furthermore, the 70% methanol extract of *Gmelina arborea* bark grown in Thrissur, Kerala, India, was reported to have *in-vitro* antioxidant activity of 124.39 mg/mL [32]. Although the reported antioxidant activities of the bark of *Gmelina arborea* by Lawrence *et al.* [32] and Attanayake *et al.* [28] were lower than that of this study, the sample collection time, geographical location, and extracting solvent may cause the differences in IC_{50} value. Singh *et al.* [30] reported that the *in-vitro* antioxidant activity of 22.7 ± 2.09 µg/mL for the methanol extract of bark of *Oroxylum indicum,* which was grown in Manipur, India was higher than that of this reporting study.

Determination of *in-vitro* Anti-inflammatory Activity: The anti-inflammatory activity **Table 3** of the standard compound and the plant extracts were expressed as the IC_{50} values in µg/mL, which is the concentration of the sample where the proteinase inhibitory activity reduced by 50%. The IC_{50} values of plant materials were determined using the graph of percentage proteinase inhibitory activity vs. concentration of the standard or sample. In evaluating *in-vitro* anti-inflammatory activity, a NSAID, diclofenac sodium was used as the reference compound [33], and it showed an IC_{50} value of 96.50 ± 0.02 µg/mL with the highest anti-inflammatory activity compared to the plant extracts analyzed within this study. However, the bark of *Gmelina arborea* and root of *Boehmeria nivea* demonstrated anti-inflammatory activity with the IC_{50} values of 119.44 ± 0.25 µg/mL and 134.75 ± 0.36 µg/mL, while the bark of *Gmelina arborea* showed the lowest anti-inflammatory activity with the IC_{50} value of 210.06 ± 2.5 µg/mL.

Correlation Analysis: The correlation of anti-inflammatory activity against TFC demonstrated a strong positive correlation with R of 0.9568 and R^2 of 0.9155 **Fig. 1**. In the correlation study, the highest R-value obtained for the correlation between the anti-inflammatory activity against the TFC further confirmed that flavonoids act as potential anti-inflammatory agents [34].

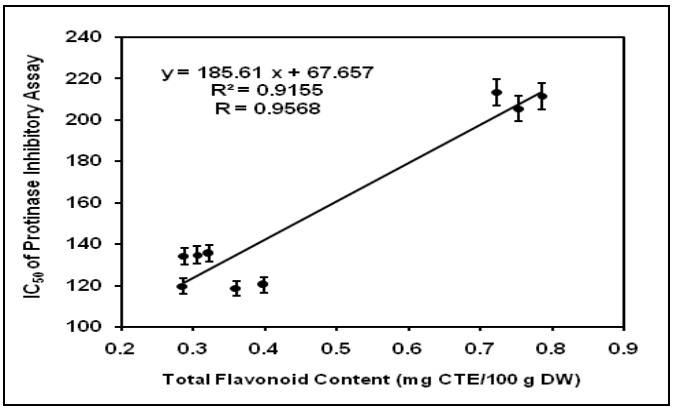

FIG. 1: CORRELATION BETWEEN THE ANTI-INFLAMMATORY ACTIVITY OF THREE SELECTED PLANT MATERIALS AND THEIR CORRESPONDING TOTAL FLAVONOID CONTENT IN METHANOL

CONCLUSION: In conclusion, the root of *Boehmeria nivea*, barks of *Gmelina arborea*, and *Oroxylum indicum* are enriched with natural antioxidants and anti-inflammatory agents. The bark extract of *Gmelina arborea*, which exhibits the highest antioxidant activity along with the highest anti-inflammatory activity, can be used as the natural source to isolate flavonoids that possess anti-inflammatory activity to treat RA.

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CONFLICTS OF INTEREST: The authors declare that there is no conflict of interest regarding this manuscript.

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