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COMPARATIVE ANTI-DIABETIC, ANTI-INFLAMMATORY, AND CHROMATOGRAPHIC SCREENING OF BIOACTIVE COMPOUNDS IN SELECTED MEDICINAL PLANTS: EXTRACTION SYSTEM EFFECTS

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Abstract

Adiantum incisum, *Alternanthera pungens* and *Trichodesma indicum* are well-known for usage in traditional folklore medicine. Therapeutic variability was assessed by *In vitro* and *In vivo* anti-diabetic and anti-inflammatory capacities. Higher yield (p<0.05) was observed for macerated samples (*A. pungens* 20.3% and *T. indicum* 54.9%) as compared to their decoction analogues (11.8% and 36.2%) with non-significant (p>0.05) yield in *A. incisum*. Macerated samples of extracts showed effective potencies with highest α-amylase inhibition (IC_{50} 101.02µg/ml) and *In vitro* anti-inflammatory capacity (IC_{50}75.89µg/ml) revealed by *A. pungens*. Pearson's correlation showed a positive association between various phenolic compounds and bioactivities. *In vivo* anti-inflammatory activity (22% inhibition compared to 31% by indomethacin) was shown by macerated *A. pungens*. Bioactive constituents and polyphenols in plant extracts were characterized by Gas Chromatography-Mass Spectrometry and High-Performance Liquid Chromatography. GC-MS identified eleven different bioactive compounds in macerated samples of *A. pungens* and twelve in decocted sample of *A. incisum.* HPLC quantified significant (p<0.05) amount of caffeic acid (26.30µg/mg), catechins (898.3µg/mg), rutin (2193.92µg/mg), kaempferol (4678.1µg/mg), gallic acid (327.04µg/mg) and quercetin (8700µg/mg) in macerated samples of *A. pungens* with significant quantity in decocted extract of *A. incisum*. Significant correlation (p<0.05) between the extraction type, phytoconstituents and their bioactivities is observed with the bioactive strength of decocted or macerated extract guided by the selection of plant choice.

Key words: *Trichodesma indicum*; *Alternanthera pungens*; *Adiantum incisum*; Polyphenols; extraction systems; Bioactive attributes

Introduction

Wild plants are an emerging source of biopharmaceuticals for their wide-ranged biological applicability (Safdar *et al.,* 2017; Rhimi *et al*., 2018; Hasnain *et al.,* 2022). Plants comprise of primary metabolites including carbohydrates, nucleic acids, fats, and amino acids as well as secondary metabolites particularly terpenoids, alkaloids and phenolics (Kabera *et al*., 2014). Phenolics have benzene rings with different hydroxyl substituents which could be in the form of simple compounds or polymers (Lin *et al*., 2016). Many studies have been performed in recent years which have established phenolic compounds as potential anti-diabetic and anti-inflammatory agents (Asgar, 2013; Mizgier *et al*., 2016). Caffeic acid, catechins, rutin, quercetin, kaempferol and gallic acid are standard phenolic compounds that possess various pharmacological properties (Oboh *et al*., 2016).

Appropriate selection of extraction methods is of crucial importance to obtain enriched amounts of different bioactive components from medicinal plants. However, no single method can be acknowledged as being solely favorable for a specific plant or a class of secondary metabolites. Effective extraction of phyto-constituents largely depends upon many factors such as chemical nature of phytoconstituents, biochemistry of plant and scientific expertise. The concentration of phenolic compounds obtained from a plant is largely affected by the employed extraction technique (Dai & Mumper, 2010). Among various methods, maceration is a traditional, inexpensive, and widely used technique for obtaining phytoconstituents from plants (Azmir *et al*., 2013). Plant material must be soaked for several days to extract bioactive components through maceration. Decoction is also extensively exploited method particularly in case of Chinese traditional medicine but entails boiling of plant material (Li *et al*., 2007). Different extraction procedures favor solubility of different type of phytochemicals like maceration is known suitable for thermolabile compound, decoction for water soluble, digestion for heat tolerant, sonication for increased permeation of solvent and serial exhaustive extraction for phytoconstituents with different polarities (Khare *et al*., 2018).

Adiantum incisum **(Ai),** *Alternanthera pungens* **(Ap)** and *Trichodesma indicum* **(Ti)** are three indigenous medicinal plants of, Pakistan. *A. incisum* (common name: Maidenhair fern) belongs to family Pteridaceae, *A. pungens* (common name: Khaki weed) belongs to family Amaranthaceae while *T. indicum* (common name: Indian borage/Andosi) belongs to family Boraginaceae. Antimicrobial, antioxidant, anti-diabetic and anti-

inflammatory characteristics of these plants have been previously established (Rai *et al*., 2016). Bio-insecticidal potential of these plants has also been studied against stored grain pests (Kazmi *et al*., 2017). These plants are being utilized by local people of Kund village for treatment of fever, cough, cold, diarrhea, skin diseases, diabetes, and inflammation (Kazmi *et al*., 2019). Nanoparticles prepared from these plants have shown potent pharmacological potential (Kazmi *et al.,* 2021). Keeping in view the immense significance of these plant species, current research was designed to investigate the inherent bioactive compounds present in plants particularly focusing upon the relation between phenolic compounds (caffeic acid, catechins, rutin, quercetin, kaempferol and gallic acid) and anti-diabetic and anti-inflammatory capacities, under the effect of two well-known extraction procedures.

Material and Methods

Sample collection and extract preparation: *Adiantum incisum*, *Alternanthera pungens* and *Trichodesma indicum* were carefully chosen due to their reported folklore medicinal importance. These three plants were collected from a small village named as Kund near Kahuta, Pakistan (Fig. 1). Identification of plants was carried out by the Department of Plant Sciences, Quaid-e-Azam University (QAU), Islamabad. Voucher specimen numbers of these plants are as followed and these were housed in herbarium of Quaid-e-Azam University, Islamabad.

Adiantum incisum (2978-ZB), *Alternanthera pungens* (1487-BA) and *Trichodesma indicum* (2564-MA).

Two types of aqueous extracts were prepared for the said collected plants. For maceration, 100g of selected plant powder (whole plant roots+stem+leaves) was soaked in 1000ml of distilled water and placed in shaking incubator (120rpm) for seven days at room temperature of 25°C±2. Solutions were filtered with gauze cloth and later with Whattman filter paper no. 41 (pore size 2.5µm). Filtrate was concentrated and stored at -20°C (Nile *et al*., 2017). For decoction (Dhanani *et al*., 2017), plant powder was boiled with distilled water (1:10, w/v) at 100°C for 30 minutes and filtered through gauze cloth and Whattman filter paper and filtrate was evaporated until dry and finally stored at -20°C. Decocted samples extracts of plants were labeled as *Adiantum incisum* (Da), *Alternanthera pungens* (Dp) and *Trichodesma indicum* (Dt).

Anti-diabetic evaluation: Antidiabetic properties of the prepared plant extracts were assessed by α-Amylase and α-Glucosidase inhibition assays according to previously reported methods (Justino *et al*., 2018). In both assays, Acarbose (Sigma) solution (100, 200, 400, 500 and 1000µg/ml) served as positive control while DMSO served as negative control. Briefly, for α-Amylase inhibition assay, sodium phosphate buffer (0.02M, pH 6.9) having Porcine pancreatic α-amylase (700-1400 units/mg protein) and plant extracts with different concentrations (100, 200, 400, 500 and 1000µg/ml) was incubated for 10min at 25°C. Starch solution (1%) was added in each reaction mixture and further incubated for 10min followed by addition of dinitrosalicylic acid (2%). Reaction mixture was placed in boiling water bath for 5 min. It was cooled, diluted with 10ml distilled water and absorbance was read at 540nm. Inhibitory activity was expressed as percentage inhibition. For α-Glucosidase inhibition assay, plant extracts with different concentrations (100, 200, 400, 500 and 1000µg/ml) and α-glucosidase solution (≥10 units/mg protein) prepared in phosphate buffer (50mM pH 6.9) were incubated at 25°C for 10mins. Then p-nitrophenyl-α-D-glucopyranoside solution was added to the buffer solution and reaction mixture was incubated at 25°C for 5 min after which absorbance was read at 405nm. Percentage inhibition was calculated using the following equation.

Anti-inflammatory potency: *In vitro* anti-inflammatory evaluation was carried out according to standard protocols reported earlier (Chirisa & Mukanganyama, 2016). For *In vitro* evaluation, different concentrations of plant extracts, acetyl salicylic acid (Sigma) and double distilled water were mixed with 0.2 ml of egg albumin (Sigma) (4% in buffer) and 2.8 ml of phosphate buffer saline (10mM). The reaction mixture was incubated at 37°C for 15 minutes and heated at 70°C for 5 minutes. After cooling, absorbance was measured at 660nm, and protein denaturation was calculated as percentage inhibition.

$$\text{Percentage inhibition} = \frac{\text{Absorbance of negative control} - \text{Absorbance of samples}}{\text{Absorbance of negative control}} \times 100$$

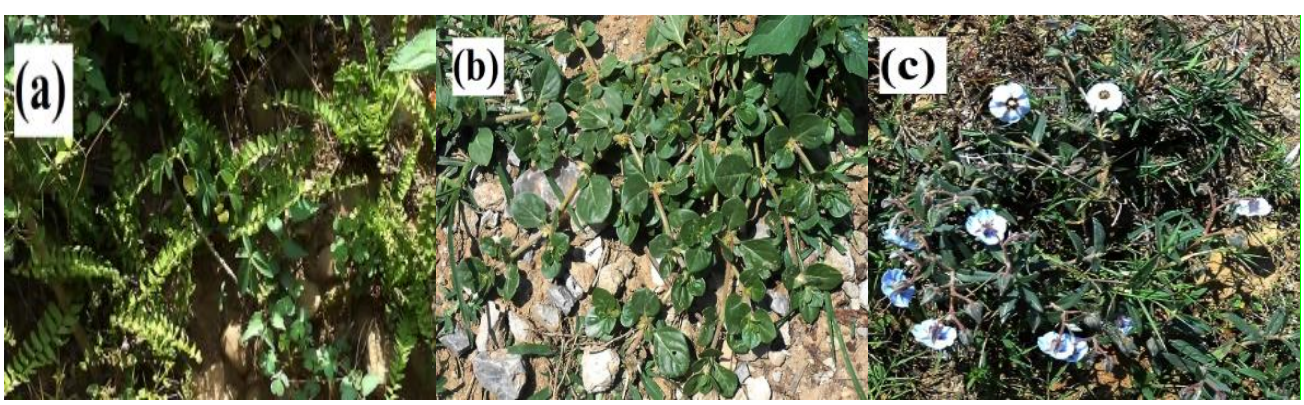


Fig. 1. *Adiantum incisum* (a), *Alternanthera pungens* (b) and *Trichodesma indicum* (c) at their natural habitat, Kund Village (Kahuta) Pakistan.

Characterization of macerated and decocted extracts: *Gas chromatography-Mass spectrometry (GC-MS)*. GC-MS [(Shimadzu GC-MS (QP-5050)] was used to examine the comparative phytoconstituents in the macerated and decocted samples. 1mg/ml of all extracts were prepared in analytical grade methanol and filtered using syringe filters (0.2 µm). Extract samples were injected into the GC portion of this instrument and the required constituents were separated according to their retention time which was then detected by mass spectroscopy. The DB-5 MS column (30m × 0.32mm i-d. 0.25µm film thickness) was used in the procedure. To carry out the analysis, 2µl of the sample was injected into GC-MS. The temperature of the injector was maintained at 250°C with an increase of 10°C/min; the oven temperature was maintained at 110°C to 280°C. Total running time for GC-MS was set to be 30 minutes and obtained chromatogram was recorded (Kim *et al*., 2016). For evaluation GC-MS (NIST 12 and 14) library was used and comparison was made between hits in library and peaks obtained in chromatograms (Kamal *et al.,* 2013).

High Performance Liquid Chromatography (HPLC): High performance liquid chromatography (HPLC) (CTO-20AC model) having UV-VIS detector was used for quantification of phenolic compounds in plant extracts which were prepared through maceration and decoction. All chemicals were purchased from Sigma (HPLC grade) and were syringe filtered (0.45 µm). Methanol served as solvents for standards and samples. Optimized conditions according to protocols (Zhang *et al*., 2013a) included solvent A to be acidified HPLC water while solvent B comprised of mixture of acetonitrile and methanol (1:2 v/v) demonstrated to be the best optimal mobile phase system The gradient volume of B was 0-50% in 0-20min, 50-100% in 20-25 min and then 100% from 25 to 30 min. For separation flow rate was maintained at 1.0ml/min while temperature of column was 25°C. Different wavelengths were used for different phenolic compounds which included 257 nm for gallic acid and rutin, 325 nm for caffeic acid, 272 nm for kaempferol, 280 nm for quercetin and 279 nm for catechins.

***In vivo* anti-inflammatory activity:** For *In vivo* experiments, ethical approval was obtained from ethical approval committee of Karachi University (Animal Study Protocol # 2018-0005). Thirty-six female Balb/c mice weighing 28-32 g were used for the determination of *In vivo* anti-inflammatory activity (David *et al*., 2014). *In vivo* anti-inflammatory potential of best identified *Alternanthera pungens* extract was studied through carrageenan-induced inflammation model in mice. Carrageenan 1% w/v (Type IV Carrageenan, Sigma) was prepared in saline (0.9% NaCl) and injected in sub plantar region of left hind paw of mice. Mice were observed for paw edema volume by using Plethysmometer (UGO Basile, Italy). Plant extract was prepared at the doses of 100 mg/kg and 150 mg/kg and dissolved in 5% DMSO using sonicator. Control groups comprised of untreated control, vehicle control (DMSO), disease control (DC), and positive control (indomethacin-Indo) (5 mg/kg) treated group. Treated groups were injected intraperitoneally with the dose of plant extract (100 and 150 mg/kg). Carrageenan was injected to Indo and the treated groups one hour after the administration of respective treatment. Paw edema was observed before carrageenan injection. Readings were taken after 1, 2, 3, 4 and 24 hours.

Statistical analysis

All experiments were carried out in triplicate followed by means represented in results with standard deviation as ±S.D. Two Way ANOVA and paired t-test was applied for assessment of significant difference (p<0.05) between extract types and phenolic compounds. Pearson correlation analysis was carried out between bioactivities and phenolic compounds of the plant extracts as quantified through HPLC using SPSS 16.0 software (IBM SPSS Software, US).

Results

Percentage yield: Aqueous extracts of three different plants were prepared by means of maceration and decoction extraction methods. Percentage yield of different extraction approaches revealed that maceration technique gave higher yield compared to decoction as depicted in (Fig. 2). Non-significant difference (p>0.05) was seen in percentage yield of *Adiantum incisum* by maceration (32.5%) and decoction (29.4%) procedures. Percentage yield of *Alternanthera pungens* and *Trichodesma indicum* significantly (p<0.05) reduced from 20.3% to 11.8% and 54.9% to 36.2% while using decoction approach.

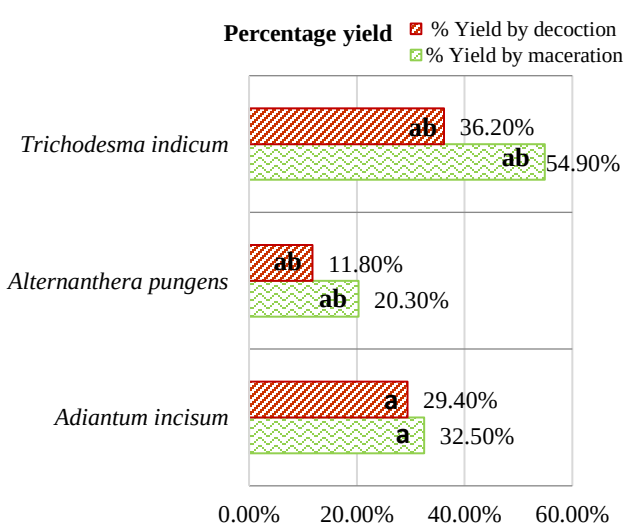


Fig. 2. Percentage yield of two different extraction approaches (similar letter denotes a subset of yield categories whose column proportion do not differ significantly from each other at the 0.05 level while different letters indicate significant difference p<0.05).

Extraction types and bioactivities: Anti-diabetic activity of all plant extracts prepared through maceration technique was significantly (p<0.05) greater compared to that of the extracts prepared through decoction method, apart from *A. incisum*. Highest α-amylase activity was observed in *A. pungens* (IC_{50}101.02µg/ml) with highest α-glucosidase activity in Ai (IC_{50}102.05µg/ml). Other extracts showed decreased activities.

A. pungens was found to be the best extract with significant anti-inflammatory potency (IC_{50} =75.89µg/ml) which is even lower than the positive control acetyl salicylic acid (IC_{50}= 129µg/ml), while the decocted analogue displayed reduced IC_{50} value i.e., 1017µg/ml. Interestingly, decocted *A. incisum* (IC_{50}678.56µg/ml) showed higher activity compared to *A. incisum* (IC_{50}894.62µg/ml). Significant difference (p<0.05) was found between the bioactivities of macerated and decocted extracts when analyzed through paired t-test (Fig. 3). Pearson correlation signifies a positive association of phenolic compounds with the bioactivities (alpha amylase, alpha glucosidase, and anti-inflammatory) of tested plant extracts (Table S1).

Effect of extraction types on bioactive constituents: Bioactive compounds in plant extracts were assessed by GC-MS and chromatograms of samples were compared with available GC-MS library hits (Table S2). Macerated samples of *A. incisum* were found rich in ketone (2-Azacyclooctanone -22.57%), ester (2,4-D isooctyl ester -14.52%) and alcohol (Cyclopropanemethanol -13.95%) which were not detected in decocted analogue. Twelve bioactive compounds were detected in decocted *A. incisum* as compared to its *macerated counterpart A. incisum* where eight compounds were identified. Macerated *A. pungens* was found rich in carboxylic acid (3-(1H-indol-3-yl)-2-[(4-nitro-2,1,3-benzoxadiazol-7-yl) amino]propanoic acid -15.77%) while decocted *A. pungens* contained ester (Methyl heptacosanoate -21.68%) in significant amount. More retention peaks were detected in macerated *A. incisum*(11) than decocted sample of *A. pungens* (7) (Fig. 4). Macerated *T. indicum* was found to be rich in ester (Methyl octadecanoate -30.08%) while decocted *T. indicum* was abundant in alkane (2-Methyltridecane -20.10%). Six retention peaks were detected in Macerated *T. indicum* than decocted *T. indicum* where only 3 peaks were identified. GC-MS detected greater compounds in macerated samples of tested extract except for *Adiantum incisum* where more compounds were revealed in decocted extract. 2D and 3D Structures of compounds identified in macerated and decocted samples of *Adiantum incisum, Alternanthera pungens* and *Trichodesma indicum* with highest percentage peaks are depicted in (Table S3).

Plant extracts prepared through maceration technique yielded higher contents of polyphenols as quantified through HPLC except for macerated sample of *A. incisum*, where increased yields of catechins, rutin and kaempferol were found in the decocted analogue (Fig. 5). Quercetin yield was found higher in decocted *T. indicum* (288.6µg/mg) in comparison to macerated *T. indicum* (97.7µg/mg). Among all six extracts, *A. pungens* was found to be the richest extract as highest content of caffeic acid (26.30µg/mg), catechins (898.3µg/mg) rutin (2193.92µg/mg) and quercetin (8700µg/mg) was quantified in this extract. Chromatograms of macerated sample of *Alternanthera pungens* and respective standards are shown in (Fig. 6) while standard curves of phenolic compounds are presented in (Fig. S1). Highest amount of kaempferol and gallic acid was observed in macerated *T. indicum* (4678.1µg/mg) and macerated *A. incisum* (327.04µg/mg) respectively. Significant difference (p<0.05) was found between different phenolic compounds groups except caffeic acid (p>0.05) using two-way ANOVA while paired t-test analysis between macerated and decocted samples revealed phenolic compounds quantity differing significantly (p<0.05) in two extraction systems tested. Chemical structures of estimated phenolic compounds in three different plants, prepared by two different extraction approaches are illustrated in (Fig. S2).

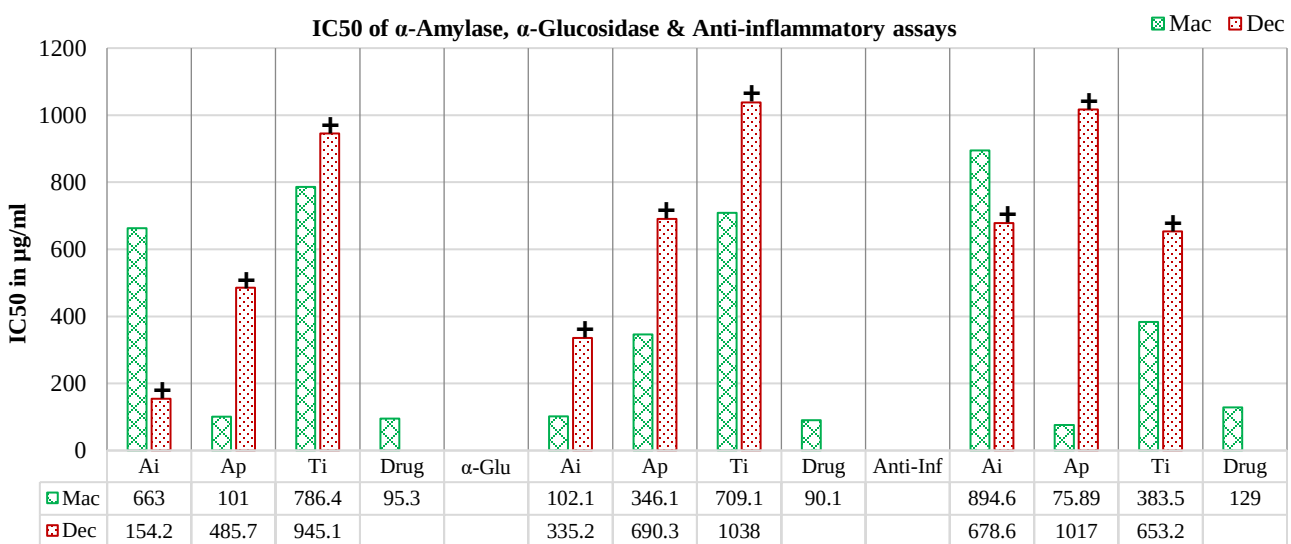


Fig. 3. Antidiabetic and anti-inflammatory potencies of macerated and decocted extracts. Drug acarbose (100-1000µg/ml) was used for α-amylase and α-glucosidase inhibition assay while drug acetyl salicylic acid (100-1000µg/ml) was used for anti-inflammatory assay. DMSO and double distilled water served as negative controls for anti-diabetic and anti-inflammatory assays respectively. Data represents an average of three replicates. Plus (+) Sign indicates significant difference (p<0.05) between macerated samples and decocted samples using paired t-test.

Table S1. Pearson's correlation between therapeutic activities (IC_{50}) and phenolic compounds of selected plants.

Phenolic compounds	Macerated sample activities			Decocted sample activities		
	α-Amy	α-Glu	Anti-Inf	α-Amy	α-Glu	Anti-Inf
	Ai			Da		
CA	0.995	-0.918	-0.572	-0.966	1.000**	0.500
CT	-0.876	1.000**	0.851	-0.966	1.000**	0.500
GA	-0.876	1.000**	0.851	-0.921	0.991	0.378
KA	-0.876	1.000**	0.851	-0.966	1.000**	0.500
RU	-0.876	1.000**	0.851	-0.966	1.000**	0.500
QU	0.102	-0.569	-0.916	-0.966	1.000**	0.500
	Ap			Dp		
CA	0.993	1.000**	-1.000**	0.974	0.982	-0.993
CT	0.993	1.000**	-1.000**	0.999*	1.000**	-.997*
GA	0.886	0.827	-0.827	0.988*	0.993	-.999*
KA	N/A	N/A	N/A	N/A	N/A	N/A
RU	0.993	1.000**	-1.000**	.999*	1.000**	-.997*
QU	0.993	1.000**	-1.000**	N/A	N/A	N/A
	Ti			Dt		
CA	-0.500	-0.500	0.485	-0.918	0.803	-0.828
CT	-0.866	-0.866	0.874	-0.500	1.000**	-0.999*
GA	-0.931	-0.931	0.925	N/A	N/A	N/A
KA	-1.000**	-1.000**	1.000*	-0.500	1.000**	-0.999*
RU	-1.000**	-1.000**	1.000*	N/A	N/A	N/A
QU	-1.000**	-1.000**	1.000*	-0.500	1.000**	-0.999*

Ai, Ap and Ti represents macerated samples of selected plants and Da, Dp and Dt represents decoction samples. N/A= Correlation cannot be computed because that plant has all 0 value. *= Correlation is significant at the 0.05 level. **= Correlation is highly significant at the 0.01 level. Negative (–) sign with values indicate negative correlation is present between phenolic compounds quantity and IC_{50} of respective plant. α-Amy= alpha amylase, α-Glu= alpha glucosidase, Anti-Inf= anti-inflammatory, CA=caffeic acid, CT= catechins, GA= gallic acid, KA= kaempferol, RU= rutin and QU= quercetin

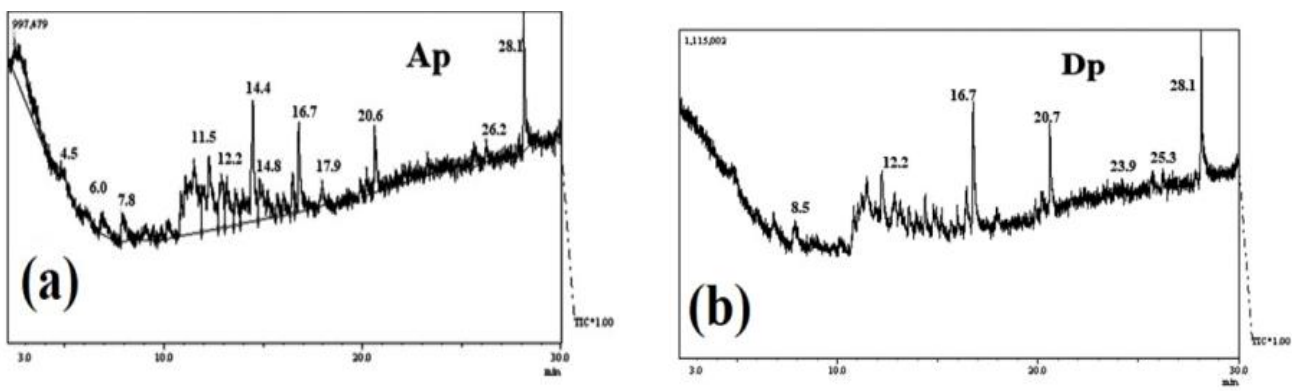


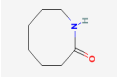
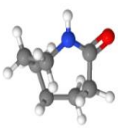
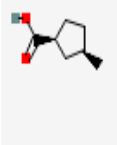
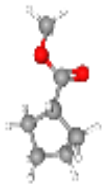
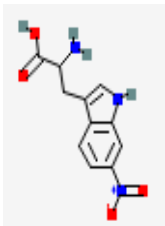
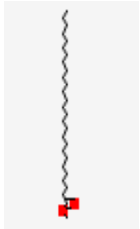
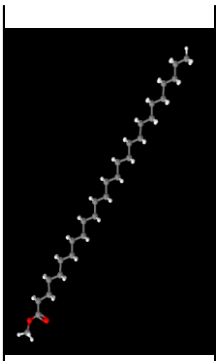
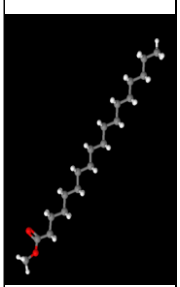
Fig. 4. GC-MS Chromatograms of macerated (Ap) (a) and decocted (Dp) (b) samples of *Alternanthera pungens*. Chromatograms of *Alternanthera pungens* were selected because it was found to be richest plant in terms of bioactivities. Retention time (in numbers) is presented above peaks. GC-MS (NIST 12 and 14) library was used, and comparison was made between hits in library and peaks obtained in chromatograms.

***In vivo* anti-inflammatory potential of macerated extract of *Alternanthera pungens*:** Due to highest *In vitro* anti-inflammatory and α-amylase inhibition activity shown by extract *A. pungens*, it was selected for screening its *In vivo* anti-inflammatory potential. Decreased plethysmometric scores by plant extracts as compared to DC (disease control) shows potential anti-inflammatory capacities (Fig. 7). Treatment with plant extract at the dose of 100 mg/kg and 150 mg/kg showed plethysmometric scores 6.8±1.0 and 6.5±0.3 respectively as compared to DC group (8.3±0.5) which after two hours of carrageenan administration, showed potential anti-inflammatory effects (p<0.01). The inflammation was gradually reduced with time; however, significant symptoms of inflammation were still observed in plant extract treated group when comparing with untreated healthy control animals suggesting treatment at higher doses may produce more significant results. Statistical analysis (ANOVA) showed significant effects of treatment with plant extract after 1h ($F(5,17)=20.34$, p<0.01), 2h ($F(5,17)=29.07$, p<0.01), 3h ($F(5,17)=28.46$, p<0.01), 4h ($F(5,17)=44.67$, p<0.01), and 24h ($F(5,17)=70.72$, p<0.01) of carrageenan administration. Post-hoc analysis showed significant induction of paw edema in DC group after one hour of carrageenan injection as compared to control animal (p<0.01) which was persistent after twenty-four hours of disease induction.

Table S2. Putative identification of compounds by GC-MS in three weeds prepared by two different extraction approaches.

	Maceration approach					Decoction approach				
	Identified compound	Class of compounds	Retention time	Molecular Formula	Peak %	Identified compound	Class of compounds	Retention time	Molecular Formula	Peak %
Adiantum incisum	2-Azacyclooctanone	Ketone	6.8	$C_7H_{13}NO$	22.57	1-Phenyl-2-propanamine	Amine	10.8	$C_9H_{13}N$	8.64
	2,4-D isooctyl ester	Ester	8.2	$C_{16}H_{22}Cl_2O_3$	14.52	1-Bicyclo[2.2.1]hept-2-ylethanamine	Amine	11.5	$C_9H_{17}N$	8.40
	Cyclopropanemethanol	Alcohol	8.8	C_4H_8O	13.95	[2-(2-Aminopropoxy)-3-methylphenyl]methanol	Alcohol	12.2	$C_{11}H_{17}NO_2$	9.38
	3-Methyltridecane	Alkane	11.7	$C_{14}H_{30}$	9.45	Cyclopropylmethanol	Alcohol	13.1	C_4H_8O	8.98
	1-Bicyclo[2.2.1]hept-2-ylethanamine	Amine	12.8	$C_9H_{17}N$	13.15	2,4-Di-tert-butyl-phenyl ester	Ester	14.3	$C_{21}H_{26}O_2$	6.64
	[2-(2-Aminopropoxy)-3-methylphenyl]methanol	Alcohol	15.2	$C_{11}H_{17}NO_2$	10.30	1-(1-adamantyl)-N-methylmethanamine	Amine	14.8	$C_{12}H_{21}N$	9.61
	1-Iodo-2-methyl undecane	Alkane	15.9	$C_{12}H_{25}I$	8.69	Tetrahydrothiophene-3-ol 1,1-dioxide	Oxide	16.0	$C_4H_8O_3S$	4.51
	2,6,11-Trimethyldodecane	Alkane	19.8	$C_{15}H_{32}$	6.84	3-(2-amino-1h-imidazol-5-yl)prop-2-enoic acid	Carboxylic acid	16.4	$C_6H_7N_3O_2$	8.84
						Methyl cyclo-pentane carboxylate	Ester	16.8	$C_{19}H_{36}O_2$	16.82
						3-Azabicyclo [3.2.2]nonane	Alkane	20.2	$C_8H_{15}N$	10.68
						Methyl 8-hydroxyoctanoate	Ester	20.6	$C_9H_{18}O_3$	4.54
						2-Amino-4-hydroxypteridine-6-carboxylic acid	Carboxylic acid	28.1	$C_7H_5N_5O_3$	2.23
Alternanthera pungens	2,3-Dimethylhexahydro-6H-pyrazolo[1,2-a][1,2,4,5]tetrazine	Tetrazine	6.0	$C_7H_{16}N_4$	10.18	2, 6-Ditert-butyl-4-methylphenyl 1-hydroxycyclopropanecarboxylate	Ester	8.5	$C_{19}H_{28}O_3$	6.25
	2-[carboxymethyl-(2,4,6-trioxo-1,3-diazinan-5-yl)amino]acetic acid	Carboxylic acid	7.8	$C_8H_9N_3O_7$	12.85	3-tert-Butyl-5-chloro-2-hydroxybenzophenone	Aromatic	12.2	$C_{17}H_{17}ClO_2$	10.1
	2-Deoxy-lyxo-hexose	Ketone	11.5	$C_6H_{12}O_5$	15.58	Methyl heptacosanoate	Ester	16.7	$C_{28}H_{56}O_2$	21.68
	3-tert-Butyl-5-chloro-2-hydroxybenzophenone	Aromatic	12.2	$C_{17}H_{17}ClO_2$	6.46	3,3'-disulfanediylbis(2-aminopropanoic acid)	Carboxylic acid	20.7	$C_6H_{12}N_2O_4S_2$	15.35
	2-Amino nonadecane	Alkane	14.4	$C_{19}H_{41}N$	6.44	2-(propan-2-ylcarbamoylamino)acetic acid	Carboxylic acid	23.9	$C_6H_{12}N_2O_3$	15.98
	(3-(1H-indol-3-yl)-2-[(4-nitro-2,1,3-benzoxadiazol-7-yl)amino]propanoic acid	Carboxylic acid	14.8	$C_{17}H_{13}N_5O_5$	15.77	4-amino-2-(2-aminopropanoylamino)-4-oxobutanoic acid	Carboxylic acid	25.3	$C_7H_{13}N_3O_4$	9.80
	Methyl 12-methyl tetradecanoate	Ester	16.7	$C_{16}H_{32}O_2$	3.22	Ditridecyl benzene-1,2-dicarboxylate	Ester	28.1	$C_{34}H_{58}O_4$	17.9
	3,5-Dipropyl-1,2,4-trioxolane	Alkane	17.9	$C_8H_{16}O_3$	4.58					
	Methyl 9-methyl heptadecanoate	Ester	20.6	$C_{19}H_{38}O_2$	2.03					
	2-Amino-4-hydroxypteridine-6-carboxylic acid	Carboxylic acid	26.2	$C_7H_5N_5O_3$	2.58					
	Ditridecyl benzene-1,2-dicarboxylate	Ester	28.15	$C_{34}H_{58}O_4$	3.76					
Trichodesma indicum	Methyl 2-hydroxy-2-methylcyclopropane-1-carboxylate	Ester	7.5	$C_6H_{10}O_3$	19.12	(3-Methyl-oxiran-2-yl)-methanol	Alcohol	9.0	$C_4H_8O_2$	9.07
	Methyl Palmitate	Ester	16.7	$C_{17}H_{34}O_2$	22.79	2-Methyl-2-nitro-1,3-propanediol	Alcohol	19.8	$C_4H_9NO_4$	12.8
	Methyl octadecanoate	Ester	19.8	$C_{19}H_{38}O_2$	30.08	2-Methyltridecane	Alkane	20.6	$C_{14}H_{30}$	20.10
	Methyl 4-ethoxybutanoate	Ester	20.7	$C_7H_{14}O_3$	7.91					
	5-Phenylocta-1,7-dien-4-ylbenzene	Aromatic	21.3	$C_{20}H_{22}$	6.42					
	1,2,3,4,5,6,7,8-Octahydrophenanthren-9-ylmethanol	Alcohol	23.8	$C_{15}H_{20}O$	7.77					

Table S3. 2D and 3D Structures of compounds identified in macerated and decocted samples of *Adiantum incisum*, *Alternanthera pungens* and *Trichodesma indicum* with highest percentage peaks. (Images copied from the open access websites www.pubchem.ncbi.nlm.nih.gov and www.chemspider.com)

Extract	Maceration approach Identified compound	2D Structure	3D Structure	Decoction approach	2D Structure	3D Structure
Ai	2-Azacyclooctanone			Methyl cyclo-pentane carboxylate		
Ap	3-(1H-indol-3-yl)-2-[(4-nitro-2,1,3-benzoxadiazol-7-yl) amino]propanoic acid			Methyl heptacosanoate		
Ti	Methyl octadecanoate			2-Methyltridecane		

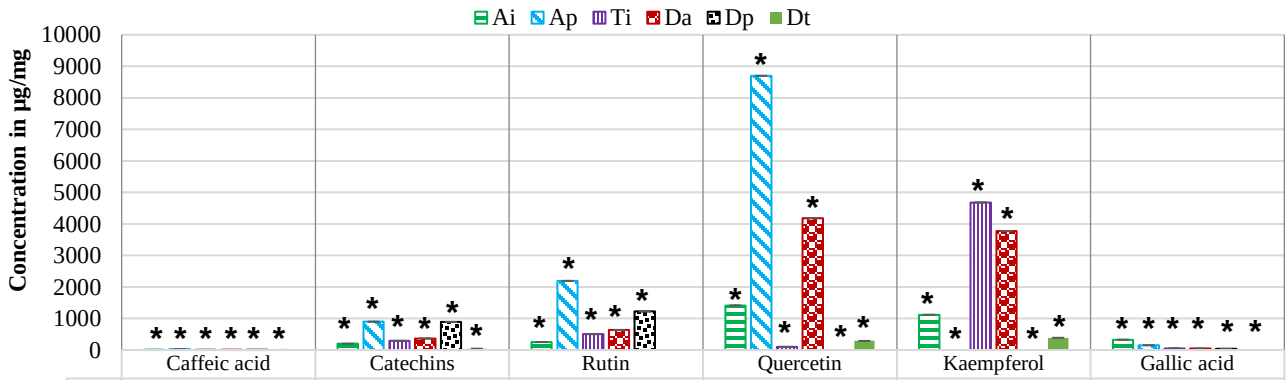
Discussion

The goal of this study was to see how two distinct extraction procedures: maceration and decoction, affected the quantity and potency of bioactive compounds isolated from three different medicinal plants. Maceration and decoction extraction processes have a significant temperature difference, with maceration taking place at room temperature and decoction requiring temperatures as high as 100°C. Moreover, maceration needs soaking, whilst decoction requires the boiling of plant matter (Zhang *et al*., 2018; Kazmi *et al*., 2019). Even though both approaches are traditional and frequently used, it is always vital to determine which extraction method is best for a specific plant. Extraction is the first stage in using a plant for pharmaceutical purposes, and it has a major impact on the extract yield and therapeutic efficacy.

For the current investigation, three different traditional medicinal plants were chosen including *Adiantum incisum*, *Alternanthera pungens,* and *Trichodesma indicum*. These plants have various significant pharmacological properties such as antioxidant, antibacterial, anti-tumor, and antifungal (Kazmi *et al*., 2019; Rastogi *et al*., 2018). Our study shows that maceration is an effective extraction technique for the selected plants except *A. incisum* whose decocted extract exhibited much higher concentration of bio-constituents and bioactivities as compared to its macerated counterpart. In *A. incisum*, the percentage yield of both extract types was almost identical. Catechins, rutin, quercetin, and kaempferol concentrations were found to be considerably greater in decocted *A. incisum* than in macerated *A. incisum*. Martins (Martins *et al*., 2014) also found that the extract generated by decoction had the largest concentration of phenolic components when compared to infusion for the hydroalcoholic extract of *Origanum vulgare*, and therefore ascribed its increased antioxidant activity to its water solubility. Aqueous decoction of mango stem bark is utilized as a nutrition supplement due to the presence of several phenolic chemicals. Our findings also imply that active phytochemicals dissolve more readily at higher temperatures, as in a decoction extraction technique, but only when suitable plants are employed. Some plants have morphological characteristics that require high temperatures for adequate dissolving of all phytoconstituents, therefore decoction extraction can be more effective for plants with harder parts (Yalavarthi, 2013). Previous studies also shows that temperature difference made a significant impact on antioxidant properties of green tea prepared by different brewing methods (Safdar *et al.,* 2016).

The decocted sample of *A. incisum* had greater anti-inflammatory and α-amylase inhibitory action than the macerated plant. As stated in the literature, phenolic compounds have effective anti-diabetic and anti-inflammatory properties (Asgar, 2013). The greater bioactivities in decocted extracts of *A. incisum* may be due to the presence of more phenolic compounds in decocted *A. incisum* as compared to macerated *A. incisum*, as shown by HPLC. The most effective IC50 for

α-amylase inhibition (101.02g/ml) and anti-inflammatory activity (75.89g/ml) was identified in *Alternanthera pungens* macerated extract, which was also found to be richest in rutin, caffeic acid, quercetin, and catechins. Quercetin is a very efficient inhibitor of α-Amylase (Meng *et al*., 2016), caffeic acid is linked to inhibition of enzymes involved in type-2 diabetes (Oboh *et al*., 2015) and rutin and catechins have well known antioxidant and anti-inflammatory potential; (Fechtner *et al*., 2017) all reminiscent of efficacious results shown by macerated *A. pungens*. The macerated extract from *A. incisum* had the greatest α-glucosidase inhibition (IC50 102.05g/ml) and was high in gallic acid (327.040.9g/ml). Gallic acid is well-known for its anti-diabetic properties.



	Caffeic acid	Catechins	Rutin	Quercetin	Kaempferol	Gallic acid
Ai	4.6	196.6	254.3	1414.26	1115.9	327.04
Ap	26.3	898.3	2193.92	8700	0	152.04
Ti	3.6	288.53	503.21	97.7	4678.1	53.49
Da	3.8	364.7	634	4183.3	3773.4	46.03
Dp	2.31	891.2	1221.9	0	0	33.73
Dt	1.9	40.9	0	288.6	386.1	0

Fig. 5. Effect of maceration and decoction on the quantification of six different phenolics. Ai, Ap and Ti represents macerated samples of selected plants *Adiantum incisum* (Ai), *Alternanthera pungens* (Ap) and *Trichodesma indicum* (Ti) and Da, Dp and Dt represents decoction samples respectively. 0 represents not detected. Standard deviation represented as error bars. Data was analyzed using post hoc test, Asterisk (*) represents significant difference (p<0.05) between groups (different phenolics). Against Da, Dp and Dt, plus sign (+) indicates significant difference (p<0.05) between macerated samples and decocted samples using t-test.

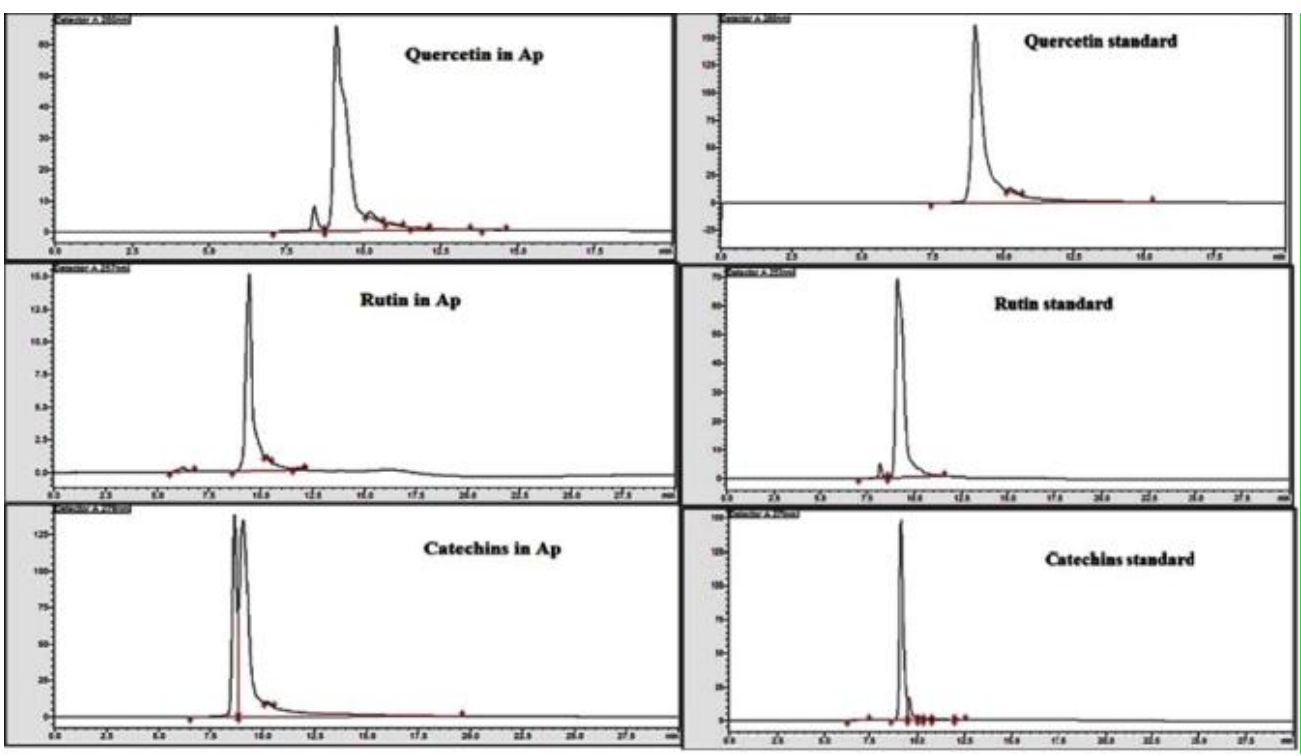


Fig. 6. HPLC chromatograms of highest phenolic compounds detected in *Alternanthera pungens* (Ap) with their respective standards. Chromatograms of *Alternanthera pungens* were selected because it was found to be richest plant in terms of bioactivities and utilized for *In vivo* experiment. Integrator of HPLC measured the peak areas and it was used against concentration for calibration curve. Linear equation was obtained using respective standard curve for each phenolic and concentrations of different phenolics in samples were calculated using straight line equation. Y=mx+c. Y= peak area of sample and x was calculated quantity of respective phenolics in samples.

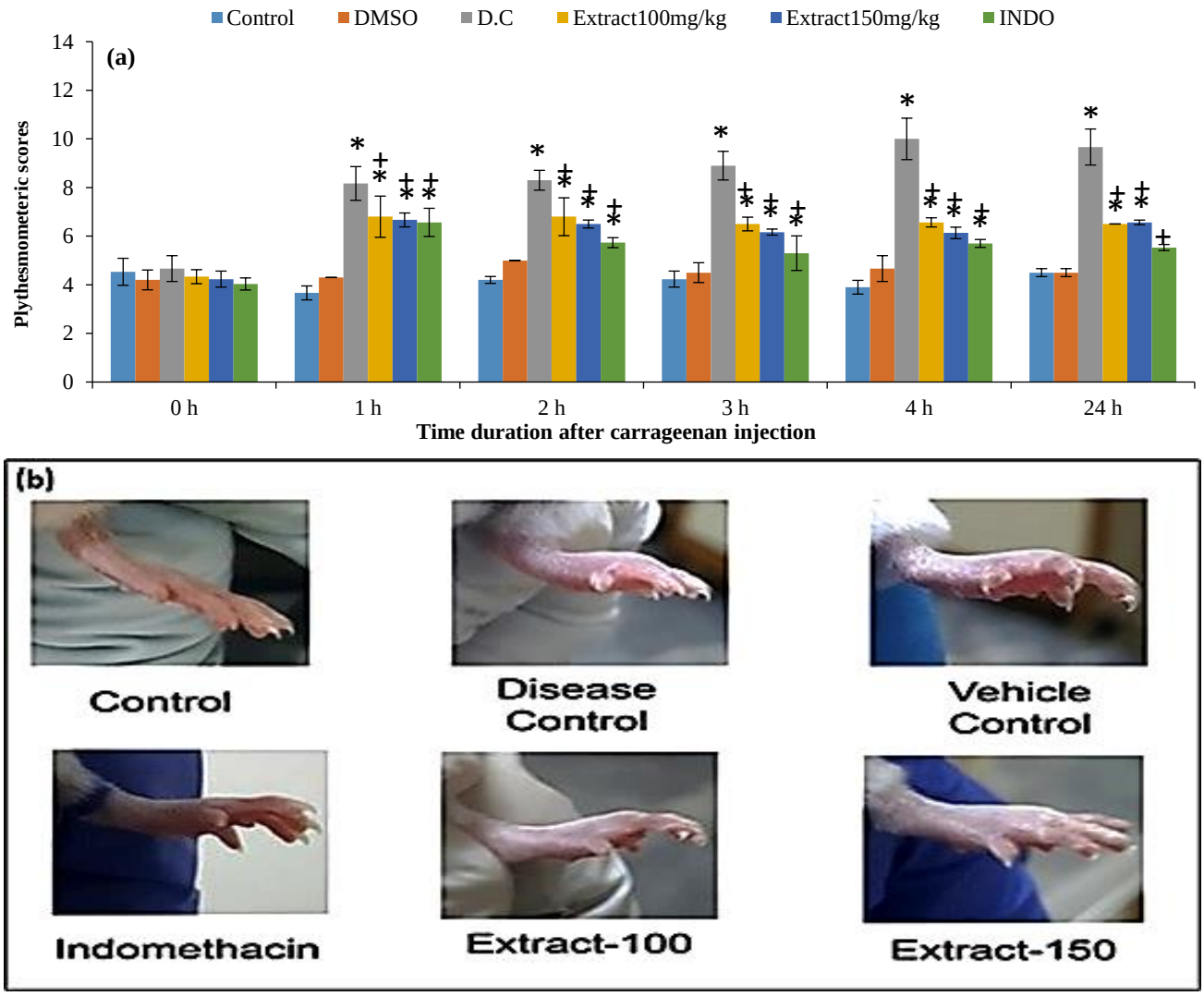


Fig. 7. Anti-inflammatory effects of plant (macerated sample of *Alternanthera pungens*) extract (a,b) in carrageenan-induced animal model of acute inflammation. Control groups comprised of untreated control, vehicle (DMSO) control, disease control (DC), and indomethacin (Indo) (5 mg/kg) treated group. Indo group served as positive control animals to compare the effectiveness of plant extract. Values are mean±SD (n=3). Data was analyzed by one-way ANOVA with Tukey's post-hoc test. P<0.01 were considered as significant. *P<0.01 as compared to control animals; +P<0.01 as compared to DC group (disease control).

The results of this study show that decoction is the preferred extraction method for *Adiantum incisum* and maceration is a better procedure for *Alternanthera pungens* and *Trichodesma indicum*. The nature of phytochemicals and their treatment in different extraction approaches causes differences in the dissolution of distinct phytoconstituents, such as for thermolabile components, maceration is appropriate, whereas decoction is suitable for water soluble phytochemicals (Khare *et al*., 2018). A prior study that identified the antioxidant activity of the neem tree (*Azadirachta indica*) using various extraction methods discovered that the decoction extract was the most efficient (Sithisarn *et al*., 2006). Another study, which investigated the pharmacokinetics of 17 distinct bioactive components using maceration and decoction processes, found that different methods of preparation had a substantial impact on the pharmacokinetics of different bio-constituents. These components were isolated from traditional Chinese medicine recipe. The recipe is prepared by using the roots of the medicinal plant *Aconitum carmichaeli,* root and rhizome of *Rheum palmatum* L., root of *Scutellariae baicalensis* and rhizome of *Coptis chinensis*. Some bio-constituents were absorbed more readily in maceration, whereas others were absorbed rapidly in decoction (Zhang *et al*., 2013b). These findings support our hypothesis that the preparation technique of plant material has a major impact on their bioactive characteristics and can dramatically affect pharmacological features by varying the dissolution property of different bioactive phyto-constituents.

The macerated plant extract of *Alternanthera pungens* showed the best *In vitro* anti-inflammatory activity (IC_{50} 75.89g/ml), highest α-amylase inhibition capacity (IC_{50} 101.02g/ml), and the richest phenolic compounds (caffeic acid, catechins, rutin and quercetin), therefore, it was also tested in an *In vivo* anti-inflammatory assay using a carrageenan-induced animal model. To our knowledge, this is the first time *Alternanthera pungens* has been tested *In vivo* for anti-inflammatory properties. Perianayagam *et al* (2006) tested a macerated sample of *Trichodesma indicum* for *In vivo* anti-inflammatory activity and discovered that the extract inhibited 48.12% of carrageenan-induced paw edema, compared to 54.32% of indomethacin standard. Our

results revealed 18% and 22% inhibition of carrageenan-induced paw edema at the dose of 100 and 150 mg/kg of Ap macerated extract in mice whereas indomethacin showed 31% inhibition. The presence of the higher quantity of polyphenols, such as caffeic acid, catechins, rutin, and quercetin, in macerated sample of *Alternanthera pungens* indicates a strong anti-inflammatory effect. Caffeic acid is involved in the suppression of several enzyme pathways, such as xanthine oxidase, cyclooxygenase, and nuclear factor-B activation, which are associated with increased inflammatory reactions. Rutin is a phenolic molecule that has been shown to be highly effective in treating chronic inflammation (Ganeshpurkar & Saluja, 2017) Catechins such as epicatechin gallate, epigallocatechin, and epigallocatechin gallate have been shown in animal models to decrease edema, bone erosion, cartilage degradation, and bone mineral density loss (Hughes *et al*., 2017). *In vitro* and *In vivo* studies on the potential of quercetin to reduce inflammation have indicated that quercetin, through diverse methods; either down regulates or inhibits numerous inflammatory pathways. The high anti-inflammation potential provided by quercetin is also due to its effect on several immune processes and antioxidant capacity (Li *et al*., 2016). Therefore, the strong *In vivo* anti-inflammatory capacity of macerated *A. pungens* can be justified by its higher quantity of active phenolic chemicals. There has been little or no research on the anti-diabetic and anti-inflammatory properties of aqueous extracts of *Adiantum incisum*, *Alternanthera pungens*, and *Trichodesma indicum* in connection to phenolic components and extraction methods till now. Our research suggests that extraction techniques for certain plant species should be optimized to get the best bioactive profile and therapeutic potential. This study clearly established significant relationship among phenolic compounds and their respective bioactivities in maceration extraction system for *Alternanthera pungens* and *Trichodesma indicum* while the decoction technique is suitable for *Adiantum incisum*. Therefore, the efficacy of bioactive components in various extraction methods is influenced by plant selection. This research suggests that extraction techniques should be optimized for each plant that is being studied and used for medicinal purposes.

Supplementary material:

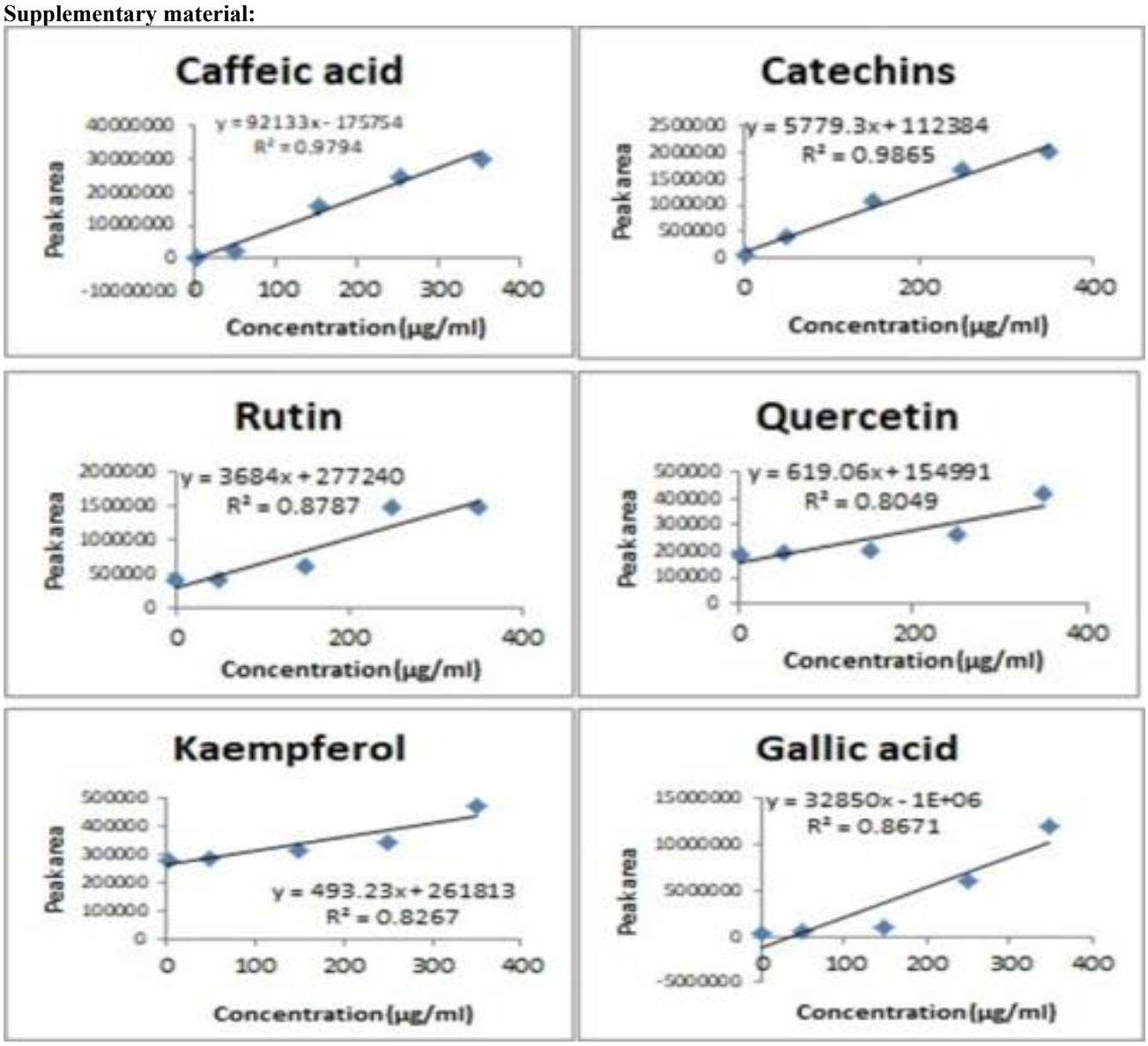


Fig. S1. Standard curves plotted for six different phenolic compounds quantified by HPLC.

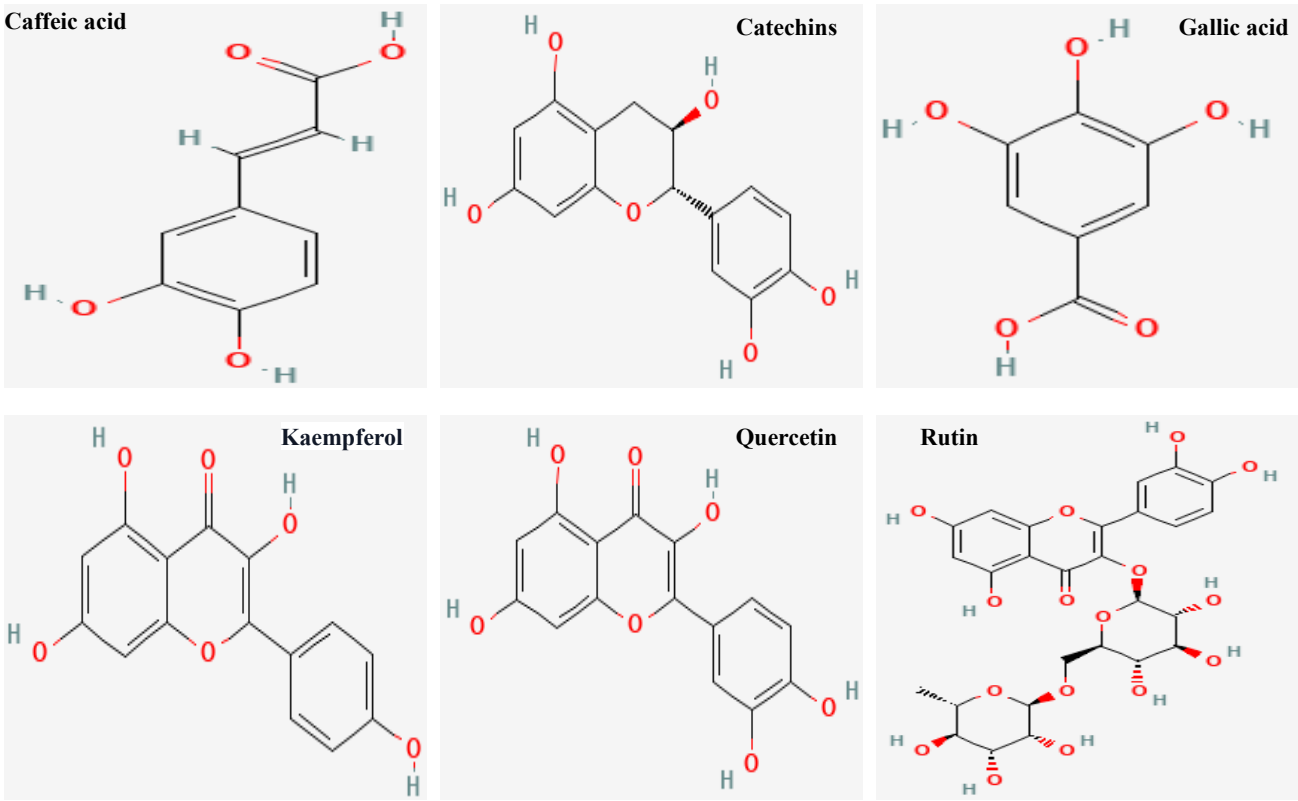


Fig. S2. Chemical structures of estimated phenolic compounds in three different plants, prepared by two different extraction approaches.

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