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TAMARIX APHYLLA; A POTENTIAL SOURCE OF ANTI-OXIDANT, ANTI-MICROBIAL AND ANTI-LEISHMANIAL METABOLITES

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TAMARIX APHYLLA; A POTENTIAL SOURCE OF ANTI-OXIDANT, ANTI-MICROBIAL AND ANTI-LEISHMANIAL METABOLITES

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Abstract

Ethnopharmacological relevance: Flora of Western Asia represents a huge reservoir of medicinal plants which can be used as a source of lead compounds for drug discovery. **Aim of the study:** Metabolite detection in, and biological activities of leaves, bark and roots extracts of *Tamarix aphylla* were determined. **Material and methods:** A comprehensive method of extraction with 14 different solvents was used to prepare samples. All samples were evaluated for their anti-microbial, anti-oxidant and anti-leishmanial potential. **Results:** Significant phenolic and flavonoid contents were found in acetone extract of leaves, ethanol extract of bark, and acetone extract of roots in particular. Ethyl acetate-ethanol extract of bark showed significant anti-oxidant activity. Acetone extract of leaves, chloroform-methanol extract of bark and ethyl acetate-ethanol extract of roots showed more than 90% inhibition of *Pseudomonas aeruginosa* in an anti-bacterial assay. In an anti-fungal assay, ethanol extract of bark showed the highest zone of inhibition against *Aspergillus niger*. The highest percent mortality of 95% was observed with chloroform-ethanol extract of roots in an anti-leishmanial assay while acetone extract of leaves showed 92% mortality. In LC-MS analysis, over 1000 metabolites were detected in bark, leaves and roots. **Conclusions:** This study has shown that multiple solvent systems applied for extraction can play a critical role in establishing medicinal potential of plants, and this specie is a potential source of pharmacologically active compounds for drug discovery.

1. Introduction

Natural-product drug discovery has experienced a revival in recent times due to the emergence of resistance against existing remedies by pathogens and the failures of other methods to yield lead compounds. The importance of natural product research in drug development provides an array of lead structures for the pharmaceutical industry. Historically, plants have been considered a source of for novel remedies and such

endeavours have had great contributions to human health and wellbeing in local communities. An estimated 80 per cent of the world's human population rely on medicines or remedies derived from plants [Verma & Singh 2008]. The flora of the Middle East and wider Asia has already proved rich with medicinal plants, however a huge percentage of plant species have not yet been studied for biologically active compounds [Gul *et al.* 2012]. Therefore drug discovery from plants remains an essential part of the search for new drugs; validating ethnobotanical data from scientifically under-explored folk plants.

Tamarix aphylla belongs to the *Tamaricaceae*, a family consisting of four genera and 120 species. *T. aphylla* is the largest known species of its genus and is native to Africa, the Middle East, India and Pakistan. Common names are Athel pine and Tamarisk [Qaiser & Perveen 2004]. A few species of *Tamarix* are used traditionally as astringent, diuretic, gargals to cure throat infections and to relieve headache [Qadir *et al.* 2014]. The bark of *T. aphylla* is an astringent tonic and is commonly used for the treatment of hepatitis, eczema, syphilis and wounds healing [Marwat *et al.* 2009]. It is also a source of chemically-diverse secondary metabolites – such as brevifolin, carboxylic acid, myricadiol, isomyricardiol, tamarixin, isoquercitrin and aphyllin [Akhlaq *et al.* 2011].

Detailed phytochemical pharmacological investigation of *T. aphylla* is limited; our study is the first to highlight the broad pharmacological potential of this species. This includes anti-oxidant, anti-fungal, anti-bacterial, anti-leishmanial, and cytotoxic potential of leaves, bark and roots of *T. aphylla*.

2. Material and methods

2.1 Collection of plants and identification

Fresh plants were collected from the peripheries of Peshawar, Khyber Pakhtunkhwa, Pakistan during June 2014. Plant samples were identified as *T. aphylla* by comparison to specimens stored in the Herbarium of Medicinal Plants, Quaid-i-azam University Islamabad, Pakistan. Leaves, bark and roots were shade dried at room temperature and crushed to make fine powder for further use.

2.2 Extract preparation

Extractions were performed using ethanol, methanol, ethyl acetate, chloroform, acetone and n-hexane, with dimethylsulfoxide (DMSO) used for sample preparation. Different solvents of varying polarity and their combinations were used, this being a total of 14 variations – seven combinations in addition to seven single compounds. These solvents were: n-hexane (H), ethyl acetate (Ea), chloroform (C), acetone (A), ethanol (E), methanol (M) and water (W). Combinations of the solvents were prepared in 1:1 ratio including ethyl

acetate-acetone (EaA), chloroform-ethanol (CE), chloroform-methanol (CM), ethyl acetate-ethanol (EaE), ethyl acetate-methanol (EaM), water-acetone (WA) and water-methanol (WM). For extraction, 50g powdered plant material of each part was weighed and soaked in 150ml of the solvents for three days and was stirred at daily intervals. Following this the solvent layer was filtered by using Whatmann's filter paper no.1, and next layer of solvent was added for two consecutive days and filtered again. A third volume of solvent was added for next two days, shaken and filtered. All filtrates were pooled and concentrated by using rotary evaporator to obtain crude extracts. The extracts were then collected in pre-weighed glass vials, labeled and stored at -20 °C for further analysis.

2.3 Percent extract recovery

Percent recovery of crude extract was calculated by using following formula:

$$\%\ Extract\ Recovery\ = \left(\frac{A}{B}\right) \times 100$$

Where A = total weight of crude extract obtained, B = total weight of plant material used for each extraction.

2.4 DPPH free radical scavenging assay

Antioxidant potential of plants extracts was determined by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) as previously described [Haq *et al.* 2012]. Each plant extract (20µl) was taken in a 96 well plate and 180µl of the DPPH reagent was added. The plate was then incubated at 37°C for about one hour. Ascorbic acid was used as a positive control while DMSO was used as a negative control. The absorbance was observed at 517 nm after 30 minutes of reaction at 37°C. Scavenging percentage was calculated by using the equation:

$$\% \, Scav. = \frac{1 - As}{Ac} \times 100$$

Where As = the absorbance of DPPH solution with sample, and Ac = absorbance of negative control. The samples showing more than 50% scavenging were further studied to calculate IC_{50}.

2.5 Antimicrobial assays

The anti-microbial activity of crude extracts of plant parts was determined by anti-bacterial (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) and anti-fungal (*Aspergillus niger*, *A. flavus* and *Fusarium solani*) assays. The antibacterial activity of crude fractions was determined by 96 well microplate reader method previously described with some slight modifications [Marasini *et al.* 2015]. Briefly, 195µl of freshly grown culture of selected bacterial strains in nutrient broth (NB) medium was added to 96 well plate already containing 5µl of the sample (4 mg/ml solution containing 20% DMSO in sterile NB). The plate was incubated (37°C, 1 hour) and optical density (OD) was taken at 600nm after incubation. The plates were subjected to second incubation at 37°C for about 24 hours. After incubation of 24 hours OD was taken using microplate reader and % inhibition was calculated.

The anti-fungal activity of each plant extract was evaluated by using the "Disc Diffusion Method" as described previously [Bibi *et al.* 2011]. Aliquots of 100 µl of each harvested fungal strain were swabbed on plates containing sterile Sabouraud dextrose agar (20 ml). Sterile filter paper discs impregnated with 5 µl (20 mg/ml DMSO) of each test extract were placed on the seeded plates. A DMSO-impregnated disc was used as negative control while those with terbinafine (a standard antifungal) served as positive control. Following incubation at 28°C for 24-48 hours, the average diameter (mm) of the zone of growth inhibition around the samples discs as well as control treated discs was measured and recorded.

2.6 Brine shrimp cytotoxicity assay

The cytotoxic effect of plant extracts was determined using brine shrimp mortality assays following a previously reported protocol [Fatima *et al.* 2015]. Brine shrimp (*Artemia salina)* eggs (Sara, Heidelberg, Germany) were hatched in shallow rectangular dish made up of two compartments (22x32 cm) filled with seawater. The compartments were separated by a wall having several holes of 2mm in diameter. One compartment was covered with aluminium foil after 24-26 hours into the start of the hatching process. The newly-hatched nauplii (brine shrimp larvae) travelled towards the enlightened compartment due to presence of light. Ten shrimps were transferred to each respective well of 96 well plates with the help of Pasteur

pipette under a 3x magnifying glass. The samples were applied in triplicate using 0.5, 1.5, 3.0µl corresponds to 200, 100, 50 and 25µg/ml respectively. Plates were incubated for 24 hours at 28°C. After incubation period the shrimps were counted to determine number of survivors percentage death was calculated. The lethal dose (LD_{50}) of the plants extract was calculated by using table curve 2D v 5.01 software.

2.7 Anti-leishmanial assay

In vitro anti-leishmanial activity the plant extracts was determined using a *Leishmania tropica* strain KWH23 culture. The strain of *L. tropica* culture was incubated for 6-7 days and cultured in Medium-199 supplemented with 10% fetal bovine serum [Fatima *et al.* 2015]. About 195 µl of harvested *L. tropica* cells were transferred onto 96-well plate and incubated at 36.5°C in humidified CO_2 (5%) incubator for 24 hours. To each well of this plate, 5 µl (50µg/ml) of sample solution containing 1% DMSO in PBS (pH 7.4) was added further diluted to required volume. Amphotericin-B was used as positive control while 1% DMSO in PBS served as negative control. Plates containing the reaction mixture were incubated for 3-5 days at 25ºC in a humidified CO_2 incubator. After 72 hours incubation, 15 µl of the test culture was visualized under light microscope for surviving promastigotes and enumerated using the improved Neubauer chamber (Marien, Germany) and percentage mortality was calculated.

2.8 LC-MS analysis

50% methanol was used as a solvent for further metabolomic analysis, using LC-MS analysis. The ZIC®-*p*HILIC (Merck Sequant) column was used for liquid chromatography, assembled within the Accela® (ThermoFisher) autosampler and pump HPLC system, according to manufacturer's instructions. An isocratic solution of 80:20 acetonitrile (stationary phase – B) : 20mM ammonium carbonate (mobile phase – A), and a 24 minute-per-sample pump method was used, as done previously by Schatschneider *et al.* (2018). The HPLC system was linked to an Exactive® (ThermoFisher) orbitrap mass spectrometer, with an ESI (electrospray ionisation) ion source. Prior to use, each time the system was calibrated with positive and negative calibration mixes. QC samples were included, and the sequence of samples injected followed the recommendations of Want *et al.* (2010), with QC samples interspaced between five randomised treatment group samples at a time, and repeated QC samples injected at the start of the run [Want *et al.* 2010]. Five mixtures (named A,B,C,D,E) of 250 standards were used for the untargeted run, as done previously [Schatschneider *et al.* 2018]. The data was analysed using XCMS [Tautenhahn *et al.* 2008],

MzMatch [Scheltema *et al.* 2011], IDEOM [Creek *et al.* 2012], and SIMCA (soft independent modelling of class analogy).

3. Results and Discussion

3.1 Percentage Extract Recovery

The percentage extract recovery was determined for different parts of plant by using maceration, and results are shown in figure 3.1.1. The yield was from bark when EaM (ethyl acetate-methanol) solvent was used. In the case of roots and leaves, the maximum extract was obtained with M (methanol) and E (ethanol) solvents. The results indicate that the polarity of extraction solvents greatly affect the yield of extract as well as biological potential, as shown in later data, corroborating previous studies [Hassim *et al.* 2015]. It has also been suggested that maceration along with combinations of different solvents could be a better choice for extraction of secondary metabolites from plant parts [Tatiya *et al.* 2011].

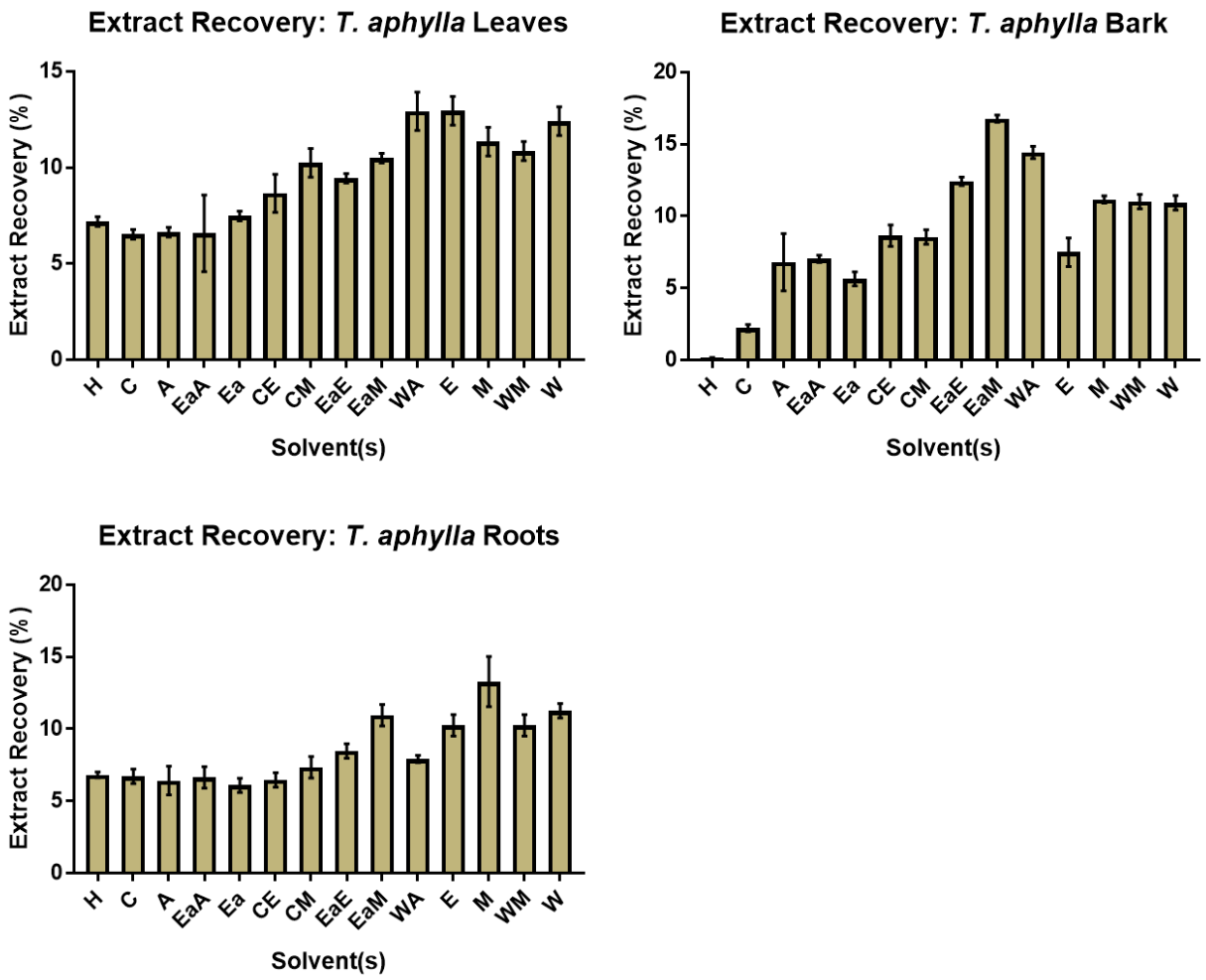


Figure 3.1.1: Percentage extract recovery, determined as proportion of dry weight, for extractions involving different solvent combinations, for leaves, bark, and roots of *T. aphylla*. Extract abbreviations for solvents: H = n-hexane, Ea = ethyl acetate, C = chloroform, A = acetone, E = ethanol, M = methanol and W = water. Combinations of the solvents in 1:1 ratio: EaA = ethyl acetate-acetone, CE = chloroform-ethanol, CM = chloroform-methanol, EaE = ethyl acetate-ethanol, EaM = ethyl acetate-methanol, WA = water-acetone and WM = water-methanol.

3.2 Free radical scavenging

Free radical scavenging potential of plant extracts was determined by using DPPH assay, and percent scavenging and IC_{50} values were determined by using table curve software. High percentage scavenging values of over 90 per cent were observed in ten bark samples (figure 3.2.1), and IC_{50} values were below 20 µg/ml (figure 3.2.2). Among the extracts of roots, six samples (A, EaA, WA, E, M and WM) showed above

90 per cent scavenging (figure 3.2.1). Extracts of leaves also showed promising results, especially the acetone extract, which had the lowest IC_{50} value of 7.32 µg/ml. These findings are consisten with previous reports about radical scavenging potential of different parts of *Tamarix* species [Drabu *et al.* 2012; Mohammedi & Atik 2011].

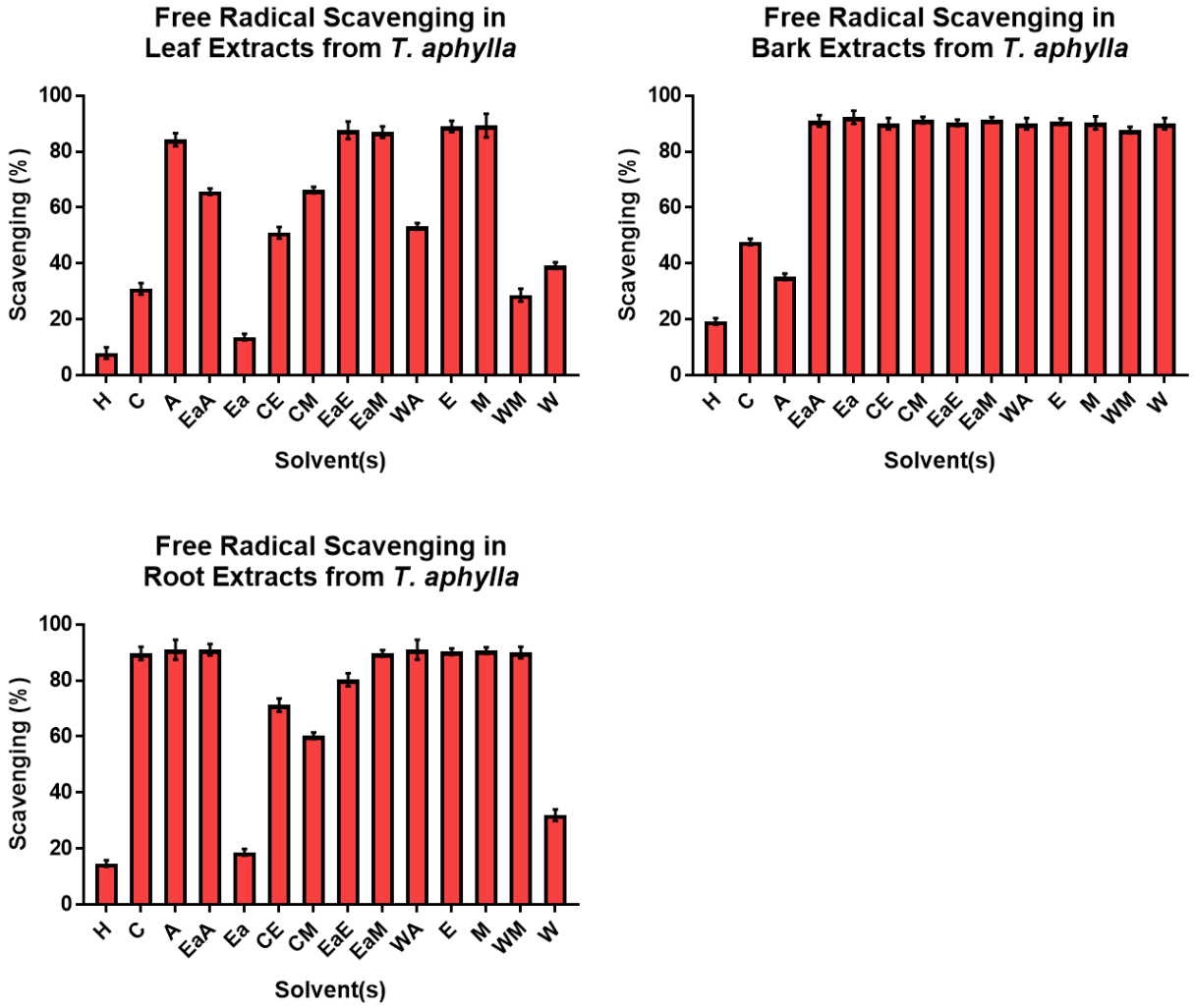


Figure 3.2.1: Percentage free radical scavenging values, as determined by DPPH assay, for extractions involving different solvent combinations, for leaves, bark, and roots of *T. aphylla*. Extract abbreviations for solvents: H = n-hexane, Ea = ethyl acetate, C = chloroform, A = acetone, E = ethanol, M = methanol and W = water. Combinations of the solvents in 1:1 ratio: EaA = ethyl acetate-acetone, CE = chloroform-ethanol, CM = chloroform-methanol, EaE = ethyl acetate-ethanol, EaM = ethyl acetate-methanol, WA = water-acetone and WM = water-methanol.

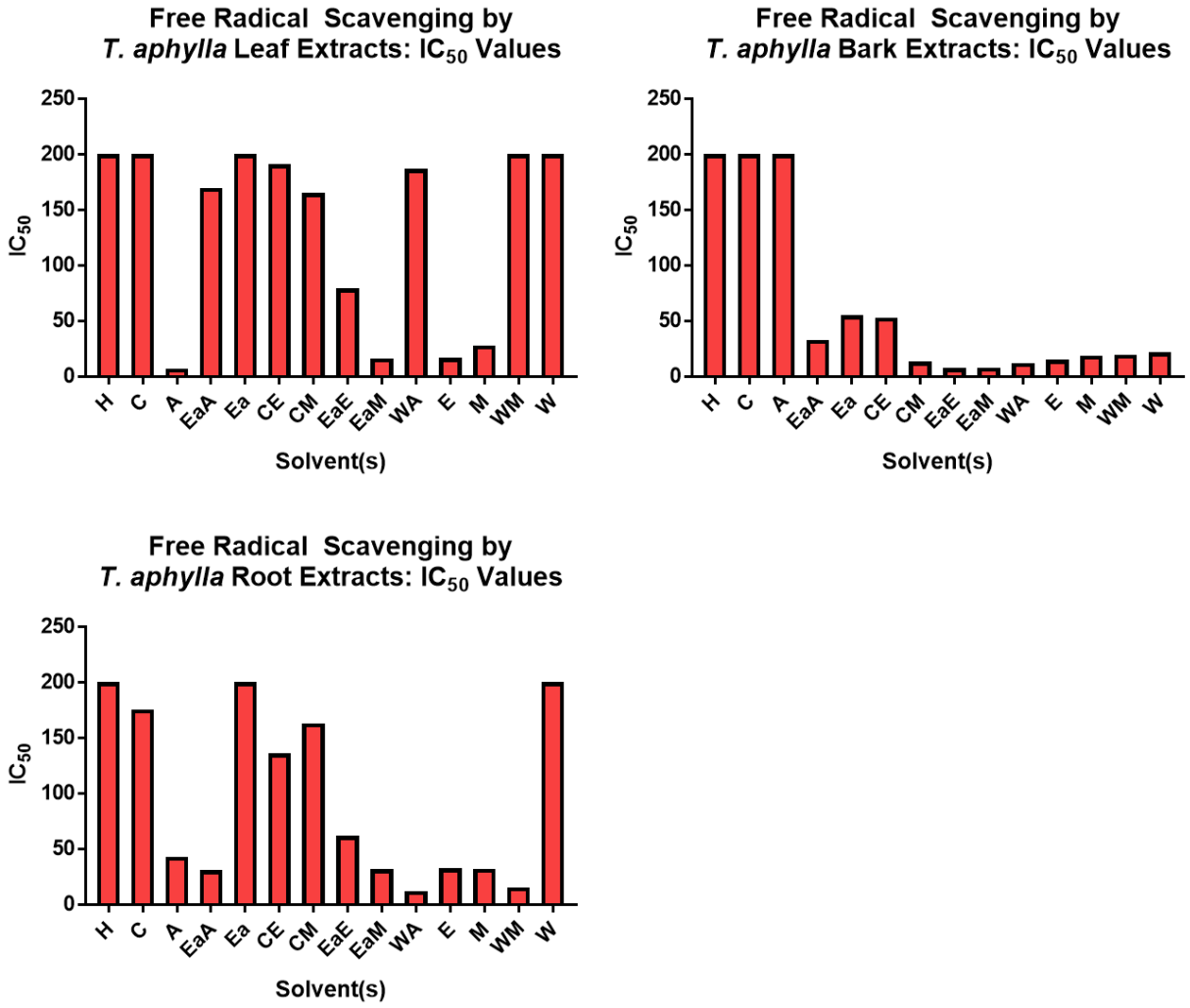


Figure 3.2.2: IC_{50} values, as determined by DPPH assay of free radical scavenging, for extractions involving different solvent combinations, for leaves, bark, and roots of *T. aphylla*. Extract abbreviations for solvents: H = n-hexane, Ea = ethyl acetate, C = chloroform, A = acetone, E = ethanol, M = methanol and W = water. Combinations of the solvents in 1:1 ratio: EaA = ethyl acetate-acetone, CE = chloroform-ethanol, CM = chloroform-methanol, EaE = ethyl acetate-ethanol, EaM = ethyl acetate-methanol, WA = water-acetone and WM = water-methanol.

3.3 Anti-bacterial and anti-fungal activities

Significant anti-bacterial activities were observed in most of the samples when tested against *Pseudomonas aeruginosa* (figure 3.3.1). The highest percentage inhibition was observed for the acetone (A) extract of leaves. Most of the extracts of roots, leaves, and bark also showed significant inhibition with a range of 70-95%. All extracts showed less than 70% inhibition when tested against *Staphylococcus aureus* (figure 3.3.2). These results are comparable with previous report of Awaad and colleagues that *Tamarix* showed significant antibacterial activity against *P. aeruginosa* [Awaad *et al.* 2014]. Additionally, other previous reports of antibacterial activity by *Tamarix* have been published [Keymanesh *et al.* 2009; Zain *et al.* 2012]. Antifungal activity was detected in extracts against *Aspergillus niger*, *Aspergillus flavus*, and *Fusarium solani* (figures 3.3.3, 3.3.4, and 3.3.5) In the antifungal assay, the highest zone of inhibition was observed with the ethanol (E) extract of bark against *A. niger* (figure 3.3.3). Root extraction samples were also active against *A. niger* with maximum zone of inhibition with the water-acetone (WA) extract. Only two samples – ethyl acetate-acetone (EaA) and ethyl acetate (Ea) extractions – of leaves showed moderate antifungal activity against *A. flavus* (figure 3.3.4) and *A. niger*. These results are comparable with previous reports about antifungal activities of *Tamarix dioica*, *T. aphylla* and *T. nilotica* against *A. flavus* and *A. fumigatus* [Awaad *et al.* 2014; Keymanesh *et al.* 2009; Zain *et al.* 2012].

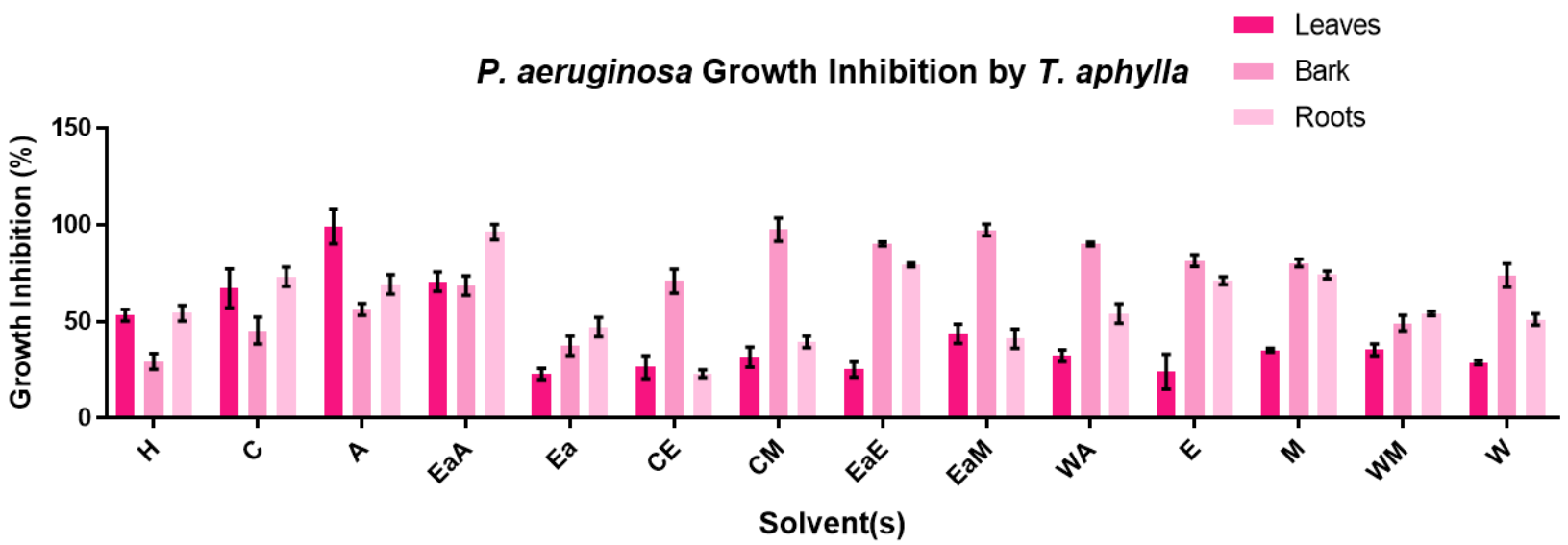


Figure 3.3.1: Antibacterial activity of *Tamarix aphylla* extracts on *Pseudomonas aeruginosa*, as measured by growth inhibition assay. Extract abbreviations for solvents: H = n-hexane, Ea = ethyl acetate, C = chloroform, A = acetone, E = ethanol, M = methanol and W = water. Combinations of the solvents in 1:1 ratio: EaA = ethyl acetate-acetone, CE = chloroform-ethanol, CM = chloroform-methanol, EaE = ethyl acetate-ethanol, EaM = ethyl acetate-methanol, WA = water-acetone and WM = water-methanol.

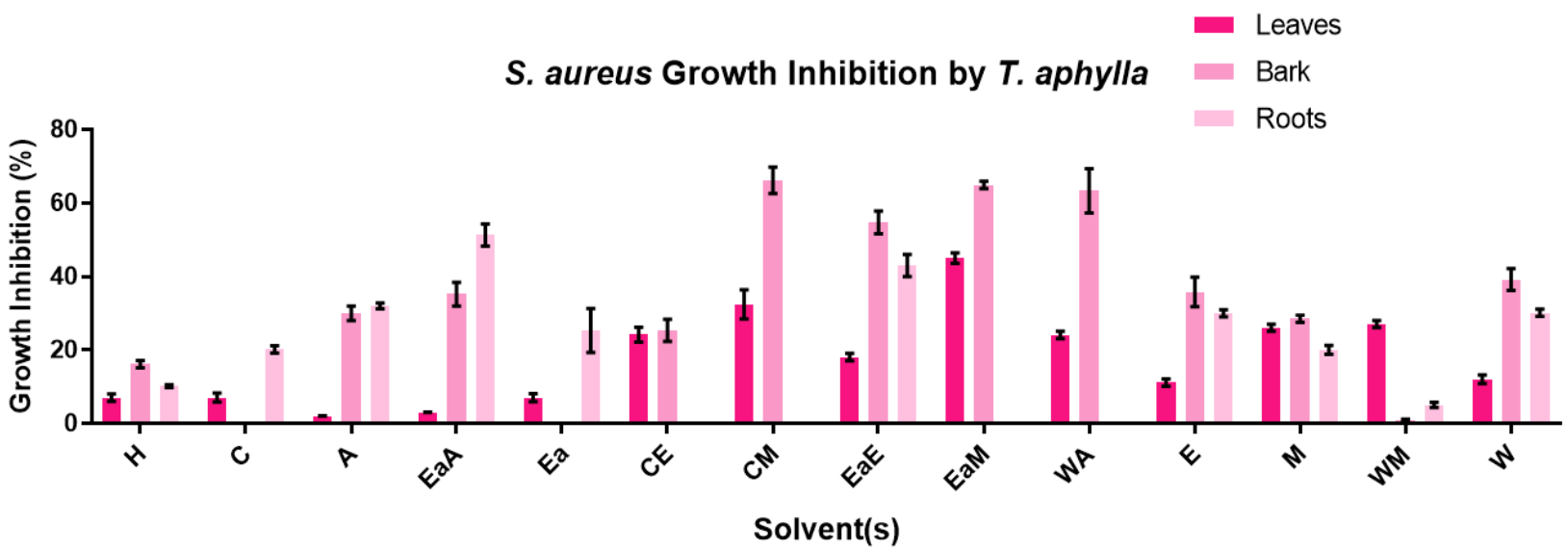


Figure 3.3.2: Antibacterial activity of *Tamarix aphylla* extracts on *Staphylococcus aureus*, as measured by growth inhibition assay. Extract abbreviations for solvents: H = n-hexane, Ea = ethyl acetate, C = chloroform, A = acetone, E = ethanol, M = methanol and W = water. Combinations of the solvents in 1:1 ratio: EaA = ethyl acetate-acetone, CE = chloroform-ethanol, CM = chloroform-methanol, EaE = ethyl acetate-ethanol, EaM = ethyl acetate-methanol, WA = water-acetone and WM = water-methanol.

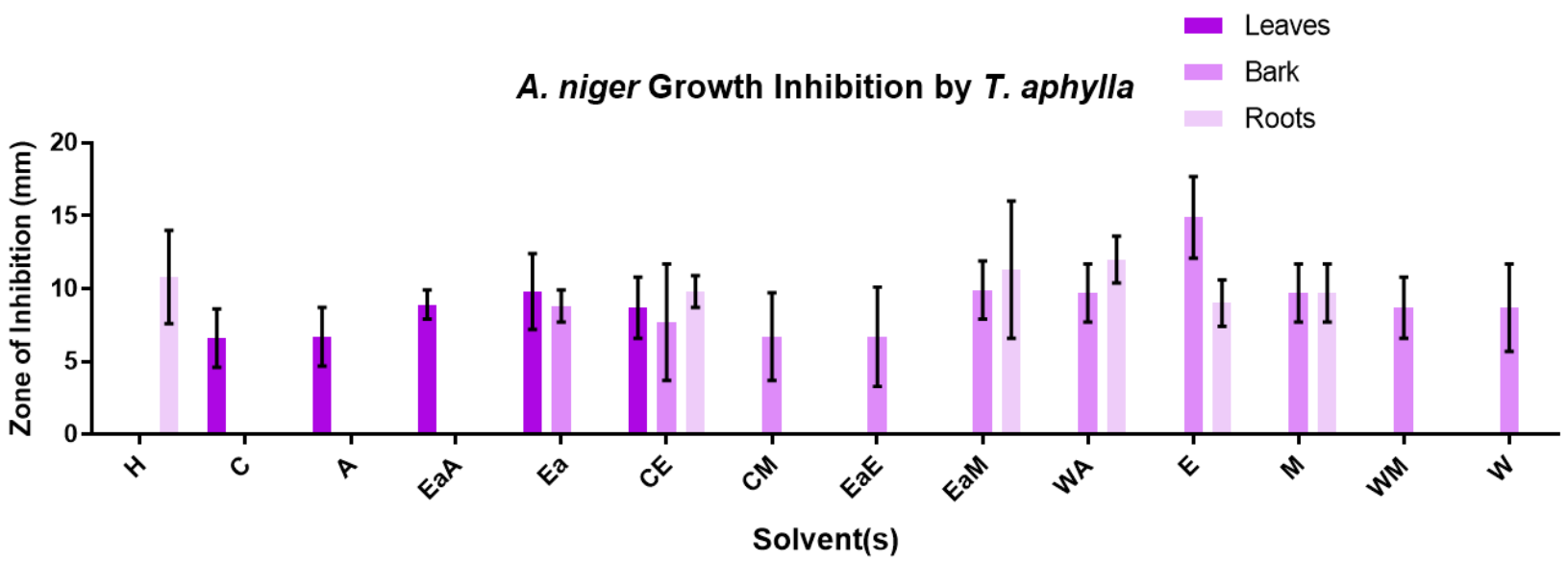


Figure 3.3.3: Antifungal activity of *Tamarix aphylla* extracts on *Aspergillus niger*, as measured by growth inhibition assay. Extract abbreviations for solvents: H = n-hexane, Ea = ethyl acetate, C = chloroform, A = acetone, E = ethanol, M = methanol and W = water. Combinations of the solvents in 1:1 ratio: EaA = ethyl acetate-acetone, CE = chloroform-ethanol, CM = chloroform-methanol, EaE = ethyl acetate-ethanol, EaM = ethyl acetate-methanol, WA = water-acetone and WM = water-methanol.

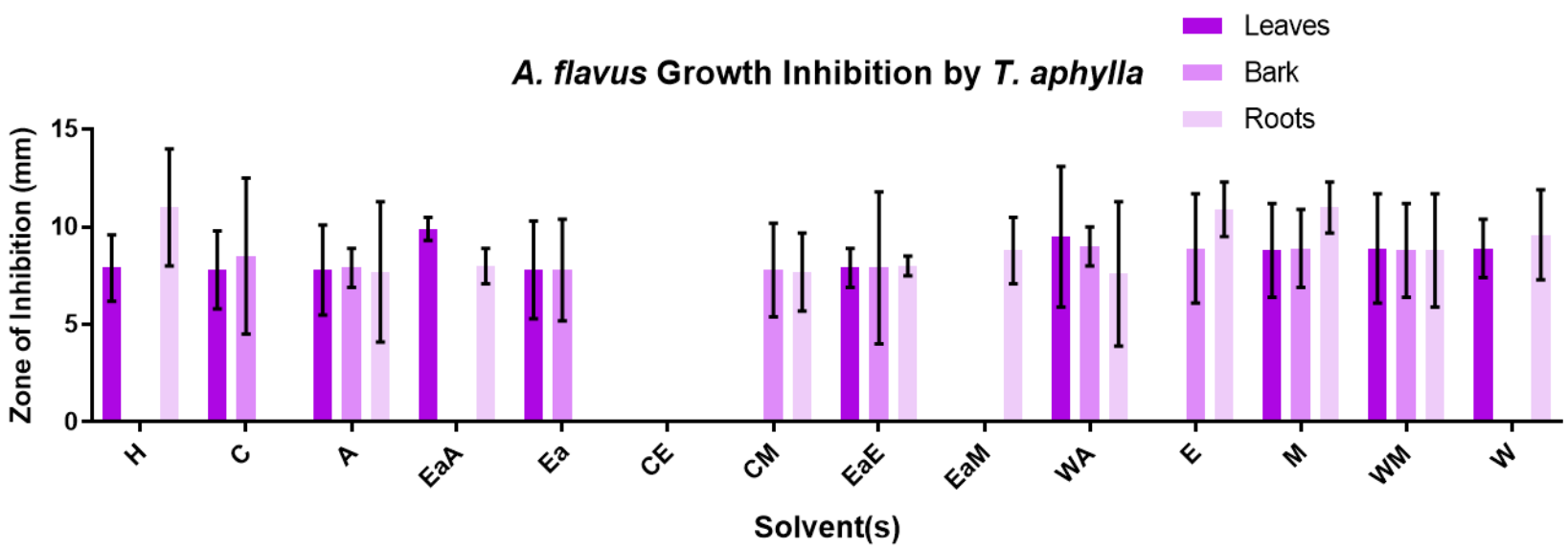


Figure 3.3.4: Antifungal activity of *Tamarix aphylla* extracts on *Aspergillus flavus*, as measured by growth inhibition assay. Extract abbreviations for solvents: H = n-hexane, Ea = ethyl acetate, C = chloroform, A = acetone, E = ethanol, M = methanol and W = water. Combinations of the solvents in 1:1 ratio: EaA = ethyl acetate-acetone, CE = chloroform-ethanol, CM = chloroform-methanol, EaE = ethyl acetate-ethanol, EaM = ethyl acetate-methanol, WA = water-acetone and WM = water-methanol.

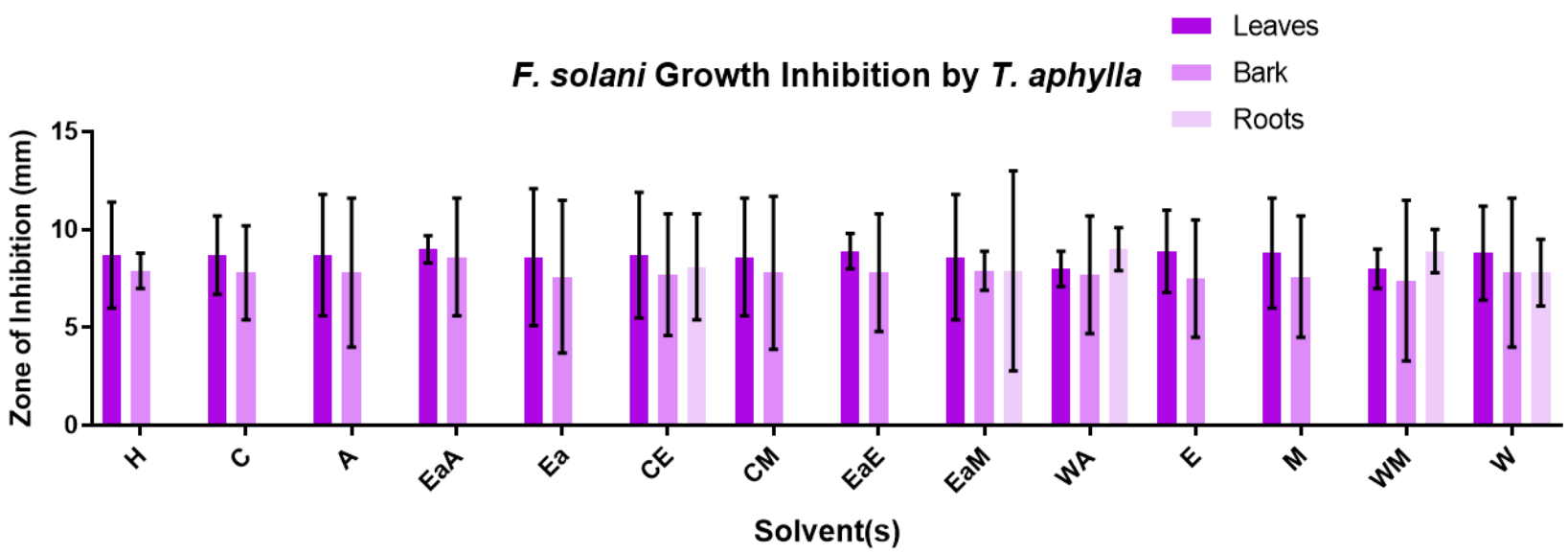
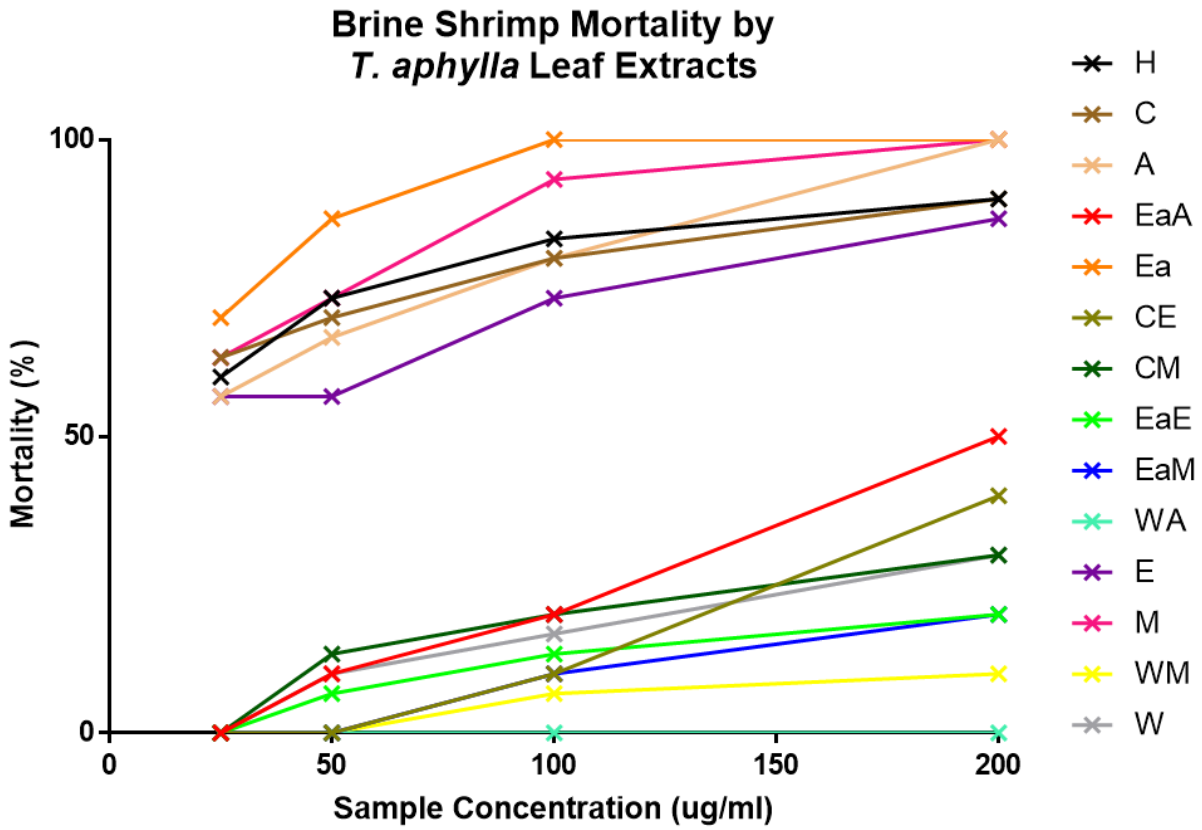


Figure 3.3.5: Antifungal activity of *Tamarix aphylla* extracts on *Fusarium solani*, as measured by growth inhibition assay. Extract abbreviations for solvents: H = n-hexane, Ea = ethyl acetate, C = chloroform, A = acetone, E = ethanol, M = methanol and W = water. Combinations of the solvents in 1:1 ratio: EaA = ethyl acetate-acetone, CE = chloroform-ethanol, CM = chloroform-methanol, EaE = ethyl acetate-ethanol, EaM = ethyl acetate-methanol, WA = water-acetone and WM = water-methanol.

3.4 Brine shrimp mortality

The results of the 24 hour brine shrimp (*Artemia salina*) cytotoxcity tests are shown in figures 3.4.1, 3.4.2, and 3.4.3, with percentage mortality and in table 3.4.1, with LD_{50} also. Extracts of leaves showed particularly strong cytotoxic effects in brine shrimp mortality assay (figure 3.4.1). This was especially the case with six of these samples (H, C, A, Ea, E and M), which showed LD_{50} values below 30 µg/ml; with lowest value for the chloroform extract of 3.83 µg/ml followed by acetone with value of 12.60 µg/ml (table 3.4.1). These findings are consistent with a previous report by Keymanesh and his colleagues about the cytotoxic potential of extracts from a plant from the same genus *Tamarix dioica* [Keymanesh *et al.* 2009]. Cytotoxic activities of plant extracts from other *Tamarix* species against *A. salina* larvae reported in previous studies have shown that it could be a potential source of anticancer compounds [Bakr *et al.* 2013; Boulaaba *et al.* 2013].



3.4.1 Brine shrimp mortality assay results for *Tamarix aphylla* leaf extracts. Extract abbreviations for solvents: H = n-hexane, Ea = ethyl acetate, C = chloroform, A = acetone, E = ethanol, M = methanol and W = water. Combinations of the solvents in 1:1 ratio: EaA = ethyl acetate-acetone, CE = chloroform-ethanol, CM = chloroform-methanol, EaE = ethyl acetate-ethanol, EaM = ethyl acetate-methanol, WA = water-acetone and WM = water-methanol.

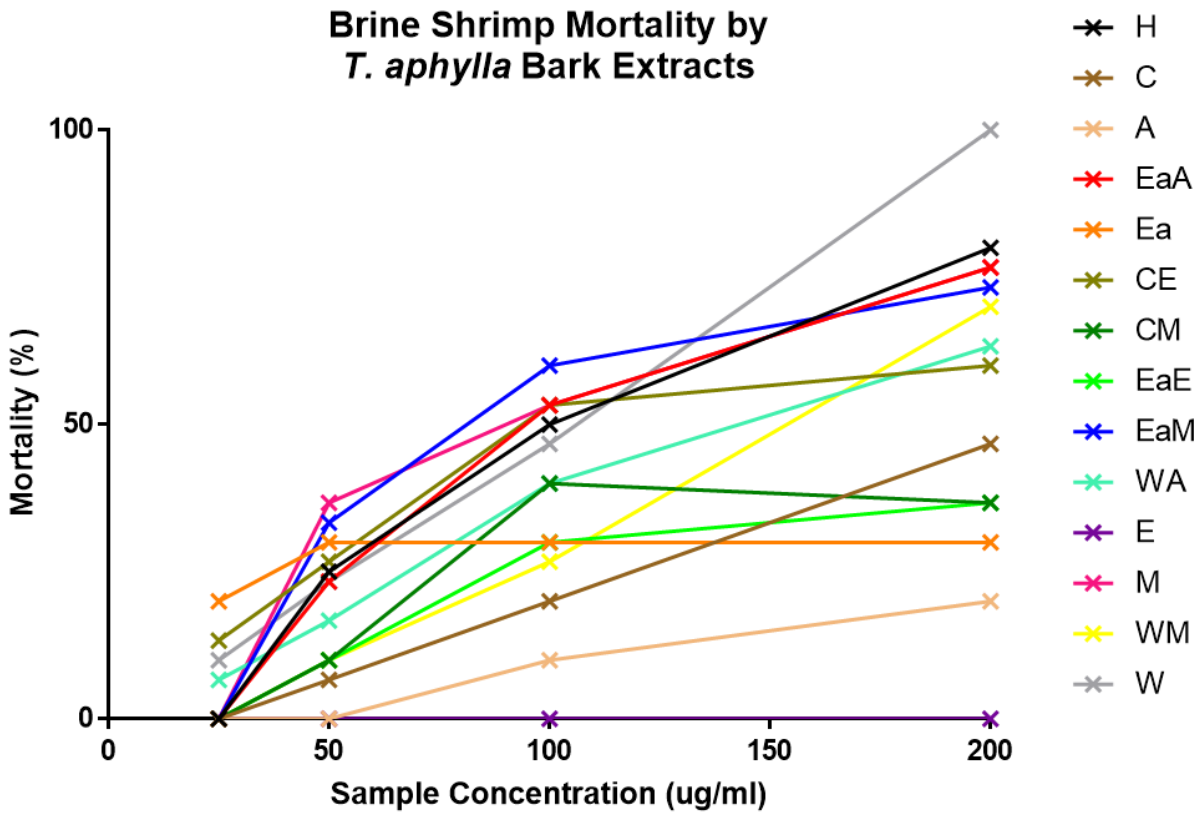


Figure 3.4.2 Brine shrimp mortality assay results for *Tamarix aphylla* bark extracts. Extract abbreviations for solvents: H = n-hexane, Ea = ethyl acetate, C = chloroform, A = acetone, E = ethanol, M = methanol and W = water. Combinations of the solvents in 1:1 ratio: EaA = ethyl acetate-acetone, CE = chloroform-ethanol, CM = chloroform-methanol, EaE = ethyl acetate-ethanol, EaM = ethyl acetate-methanol, WA = water-acetone and WM = water-methanol.

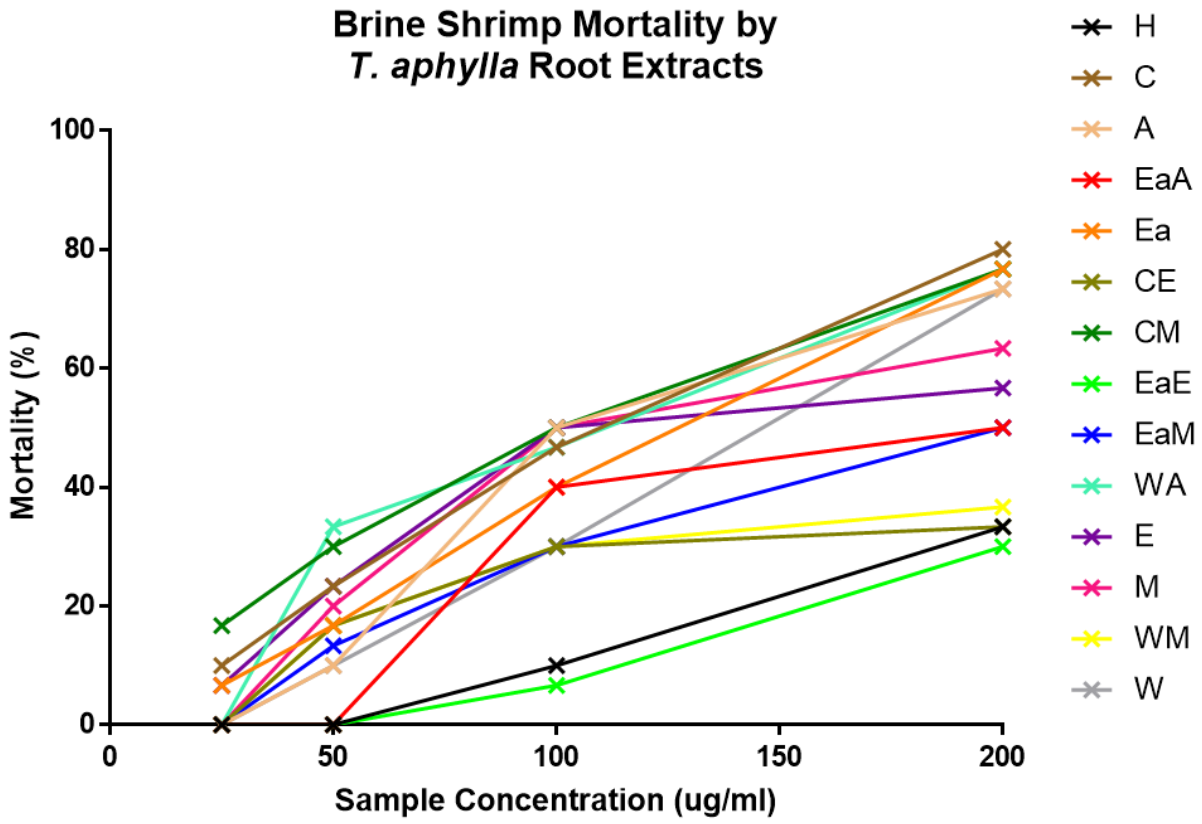


Figure 3.4.3 Brine shrimp mortality assay results for *Tamarix aphylla* root extracts. Extract abbreviations for solvents: H = n-hexane, Ea = ethyl acetate, C = chloroform, A = acetone, E = ethanol, M = methanol and W = water. Combinations of the solvents in 1:1 ratio: EaA = ethyl acetate-acetone, CE = chloroform-ethanol, CM = chloroform-methanol, EaE = ethyl acetate-ethanol, EaM = ethyl acetate-methanol, WA = water-acetone and WM = water-methanol.

Table 3.4.1: Brine shrimp mortality assay of various solvent extracts of three different parts of *T. aphylla.* Extract abbreviations for solvents: H = n-hexane, Ea = ethyl acetate, C = chloroform, A = acetone, E = ethanol, M = methanol and W = water. Combinations of the solvents in 1:1 ratio: EaA = ethyl acetate-acetone, CE = chloroform-ethanol, CM = chloroform-methanol, EaE = ethyl acetate-ethanol, EaM = ethyl acetate-methanol, WA = water-acetone and WM = water-methanol.

Solvent(s)	Leaves (% mortality and LD_{50})					Bark (% mortality and LD_{50})					Roots (% mortality and LD_{50})				
	200 µg/ml	100 µg/ml	50 µg/ml	25 µg/ml	LD_{50}	200 µg/ml	100 µg/ml	50 µg/ml	25 µg/ml	LD_{50}	200 µg/ml	100 µg/ml	50 µg/ml	25 µg/ml	LD_{50}
H	90	83.3	73.3	60	15.8	80	50	25	0	10.28	33.33	10	0	0	>200
C	90	80	70	63.3	3.83	46.7	20	6.67	0	>200	80	46.7	23.3	10	110
A	100	80	66.7	56.7	12.6	20	10	0	0	>200	73.33	50	10	0	100
EaA	50	20	10	0	>200	76.7	53.3	23.3	0	92.7	50	40	0	0	200
Ea	100	100	86.7	70	14.8	30	30	30	20	>200	76.67	40	16.7	6.67	123.6
CE	40	10	0	0	>200	60	53.3	26.7	13.3	95.29	33.33	30	16.7	0	>200
CM	30	20	13.3	0	>200	36.7	40	10	0	>200	76.67	50	30	16.7	100
EaE	20	13.3	6.67	0	>200	36.7	30	10	0	>200	30	6.67	0	0	>200
EaM	20	10	0	0	>200	73.3	60	33.3	0	132.3	50	30	13.3	0	199.77
WA	0	0	0	0	>200	63.3	40	16.7	6.67	132.3	76.67	46.7	33.3	0	96.27
E	86.7	73.3	56.7	46.7	31.7	0	0	0	0	>200	56.67	50	23.3	6.67	100
M	100	93.3	73.3	63.3	13.4	76.7	53.3	36.7	0	78.26	63.33	50	20	0	100
WM	10	6.67	0	0	>200	70	26.7	10	0	156.1	36.67	30	16.7	0	>200
W	30	16.7	10	0	>200	100	46.7	23.3	10	105.7	73.33	30	10	0	147.19

3.5 Anti-leishmanial activity

Leishmaniasis is reported throughout tropical regions [Khoshzaban *et al.* 2014]. The response of *T. aphylla* extracts against cutaneous leishmaniasis was determined here, through a mortality assay, the results of which are shown in figure 3.5.1. Eight extracts of roots showed highly potent activity with values of mortality over 80 per cent. Out of these, the chloroform-ethyl acetate (CE) extract showed the highest percentage mortality. Among leaf samples, the acetone (A) extract showed the highest percentage mortality with value of 92 per cent, followed by chloroform (C) extract with value of 91 per cent (figure 3.5.1). Chloroform and acetone extracts of bark also showed high potency to the parasite. Previously there have been relatively few reports about anti-leishmanial study of plant parts [Hamid *et al.* 2012]. Hence this study presents an early overview of the anti-leishmanial potential of various extracts from different plant parts of this remarkable species.

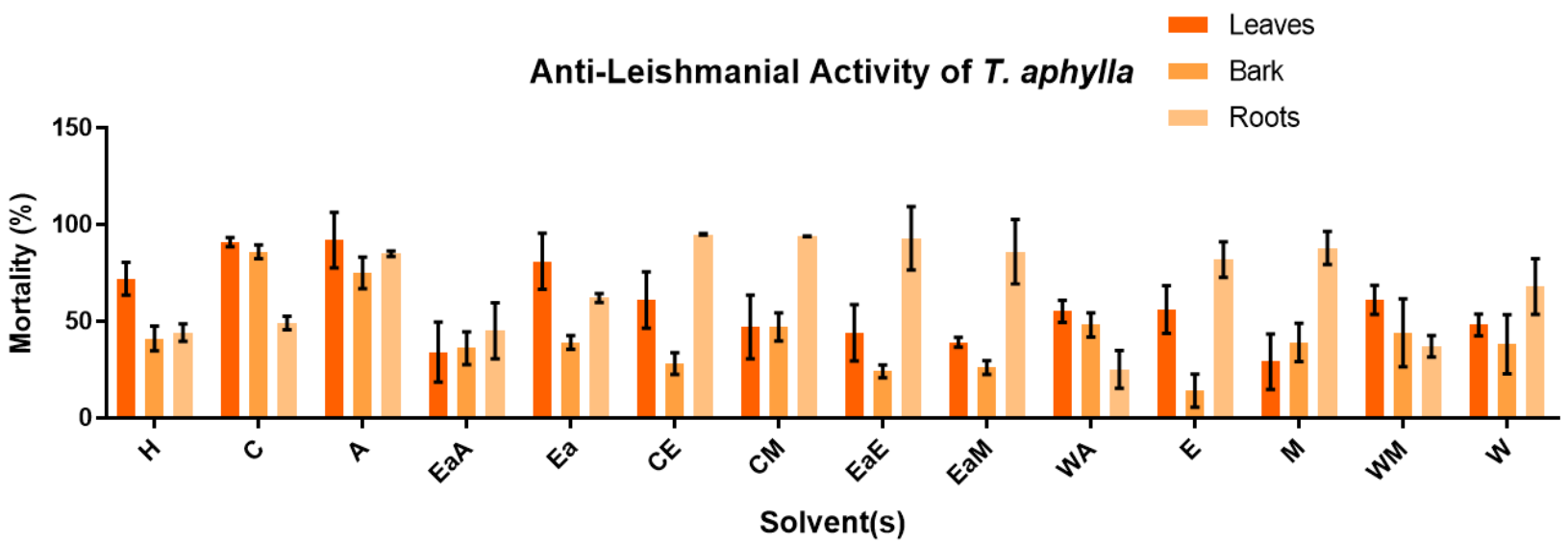


Figure 3.5.1: Anti-leishmanial activity of extracts from *Tamarix aphylla*, as determined by mortality assay. Extract abbreviations for solvents: H = n-hexane, Ea = ethyl acetate, C = chloroform, A = acetone, E = ethanol, M = methanol and W = water. Combinations of the solvents in 1:1 ratio: EaA = ethyl acetate-acetone, CE = chloroform-ethanol, CM = chloroform-methanol, EaE = ethyl acetate-ethanol, EaM = ethyl acetate-methanol, WA = water-acetone and WM = water-methanol.

3.6 LC-MS analysis

Numbers of detected metabolites from leaf, bark, and root extracts are shown in figures 3.6.1 and 3.6.2. Overall, totals of 1433, 1457, and 1140 metabolites were detected in leaf, bark and root extracts respectively (figure 3.6.2), including those involved in amino acid metabolism, biosynthesis of secondary metabolites, carbohydrate metabolism, lipid metabolism, metabolism of cofactors and vitamins, nucleotide metabolism, peptides and others (figures 3.6.1 and 3.6.2). PCA (Principal component analysis, figure 3.6.3), and OPLS-DA (orthogonal partial least squares analysis, figure 3.6.4) performed by SIMCA (soft independent modelling of class analogy) show consistently separate clustering of leaf, bark, root, and also flower extracts, indicating distinct metabolic profiles for each of the plant parts. The tight clustering of QC samples in the center of the principal component analysis plot (figure 3.6.3) shows a strong consistent performance of the mass spectrometer throughout the run.

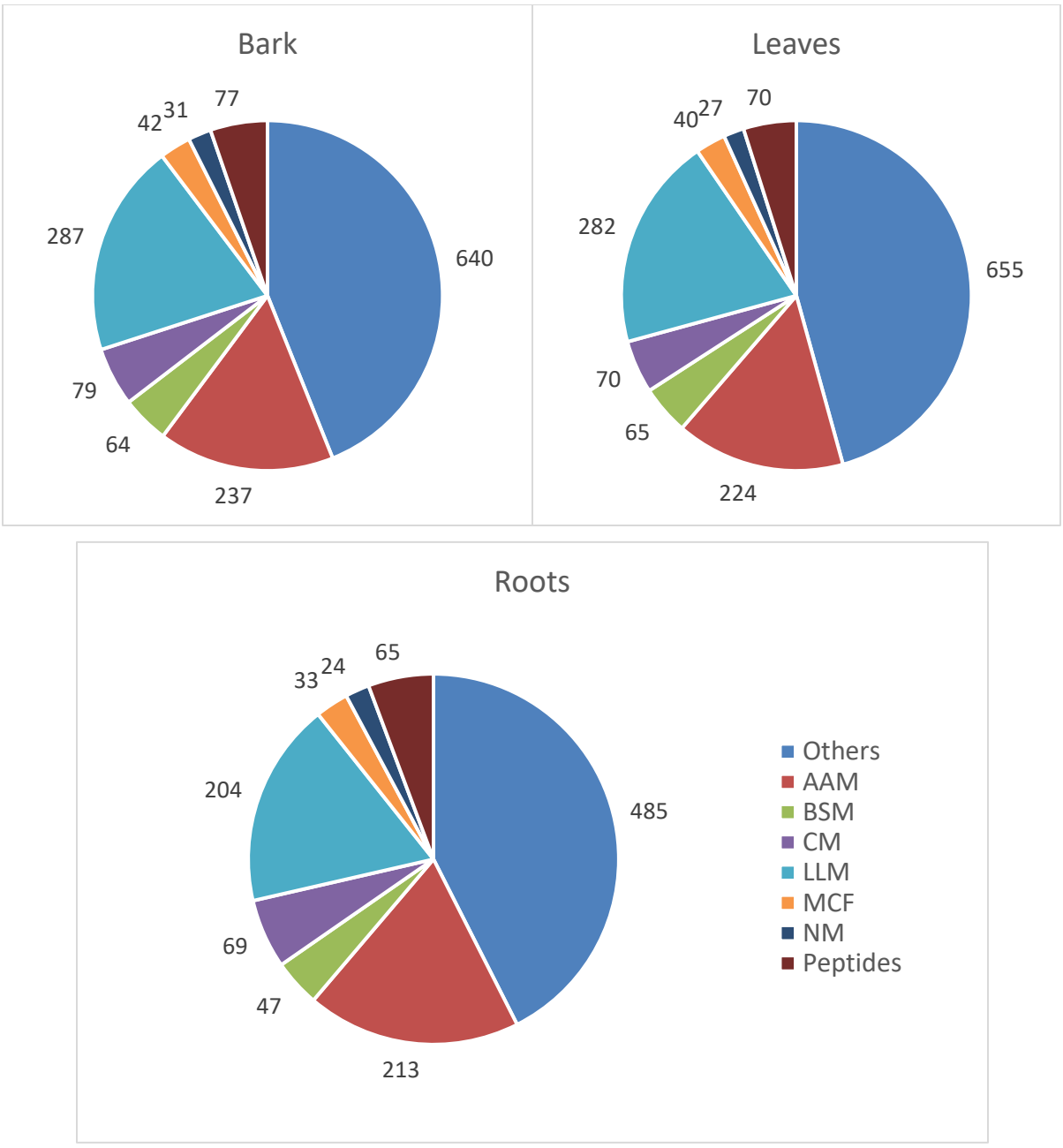


Figure 3.6.1: Pie charts showing numbers of metabolites detected in the LC-MS analysis of leaf, bark and root samples. Abbreviations: AAM = amino acid metabolism; BSM = biosynthesis of secondary metabolites; CM = carbohydrate metabolism; LLM = lipids and lipid metabolism; MCF = metabolism of cofactors and vitamins; NM = nucleotide metabolism.

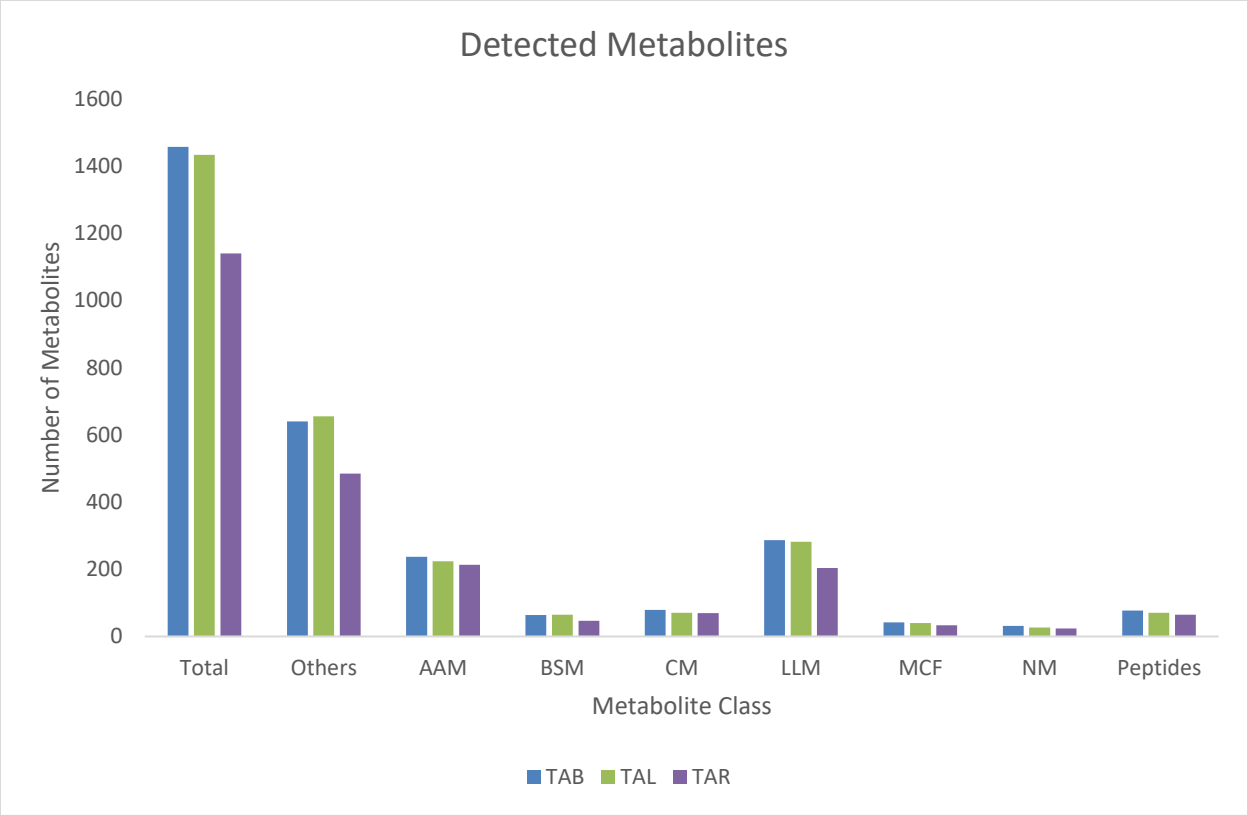


Figure 3.6.2: Metabolites detected in the LC-MS analysis of leaf, bark and root samples. Abbreviations: AAM = amino acid metabolism; BSM = biosynthesis of secondary metabolites; CM = carbohydrate metabolism; LLM = lipids and lipid metabolism; MCF = metabolism of cofactors and vitamins; NM = nucleotide metabolism.

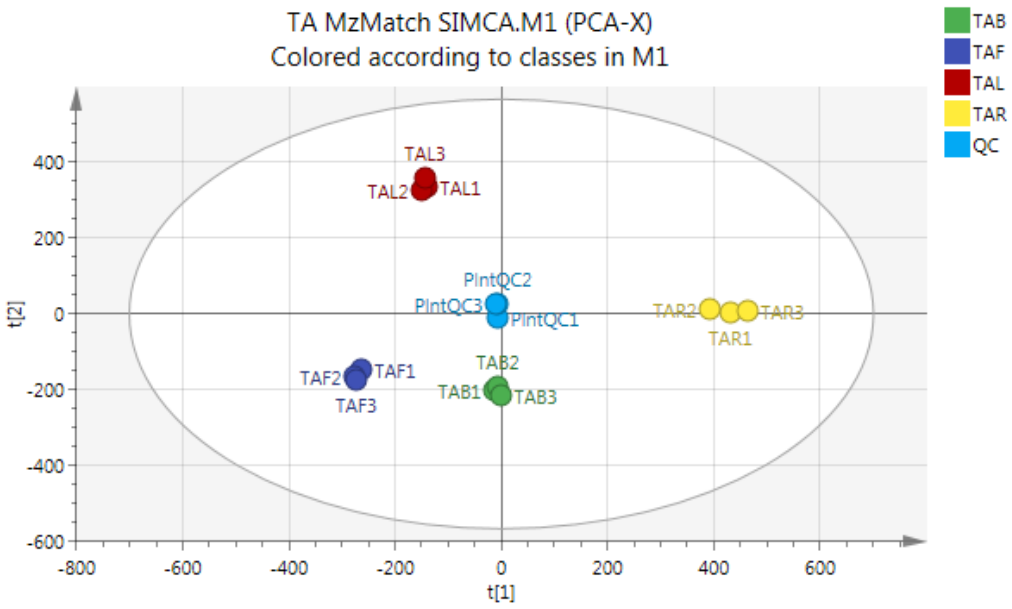


Figure 3.6.3: Principal component analysis (PCA) plot, constructed using SIMCA, showing extract samples. Abbreviations: TAB = bark extracts; TAF = flower extracts; TAL = leaf extracts; TAR = root extracts.

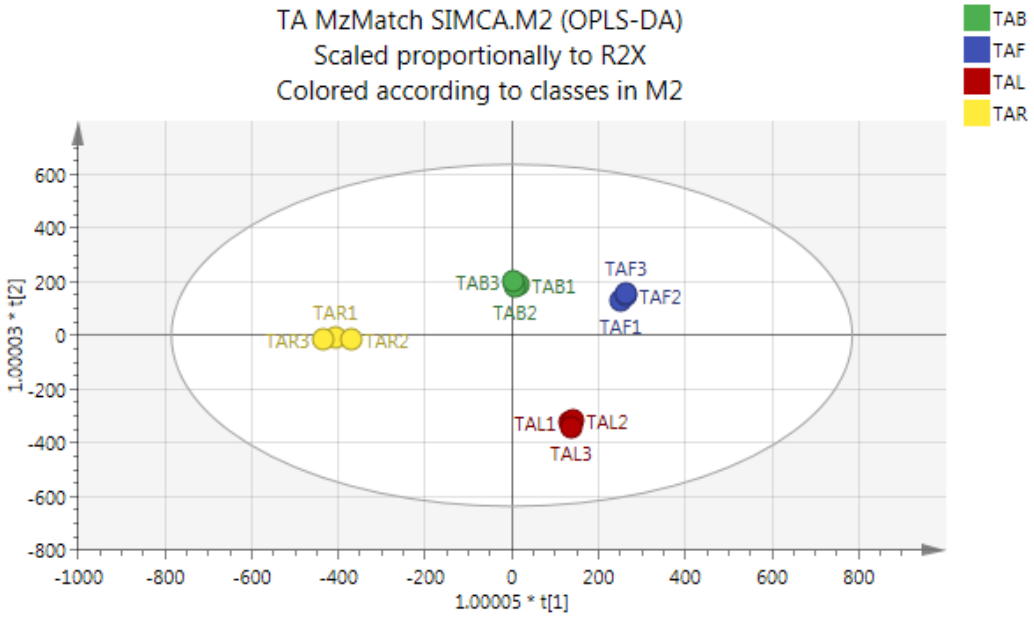


Figure 3.6.4: Orthogonal partial least squares analysis (OPLS-DA) plot, constructed using SIMCA, showing extract samples. Abbreviations: TAB = bark extracts; TAF = flower extracts; TAL = leaf extracts; TAR = root extracts.

4. Conclusion

Extracts from *Tamarix aphylla* show promising effects, indicating a potential for the development of drugs following further research from lead compounds. Antibacterial, antifungal, anti-leishmanial, and antioxidant effects have all been shown the data presented in this study. Brine shrimp mortality assay data confirms varying levels of cytotoxicity in animal cells, although no targeted approach has yet been undertaken with this plant or related species to ascertain potential for the development of new cancer drugs. LC-MS analysis using complex standards has proven a good method for detecting a wide range of metabolites in *T. aphylla*, and further work with this and other analytical methods could be used to identify candidate compounds for the biological activities described. Using a polarity range-based selection of extraction solvents can be useful strategy for isolating these compounds also – as comparing between extracts can give clues to the chemical structures and properties of compound(s) involved in biological activities. Studies like this one play an important preliminary role in identifying the possible pharmaceutical and therapeutic properties of products from plants and other natural resources, making use of this rich and largely-unexplored reservoir.

REFERENCES

Akhlaq PM, Mohammed A. 2011. New phenolic acids from the galls of *Tamarix aphylla* (L.) Karst. International Research Journal of Pharmacy. 4, 222-225.

Awaad AS, Zain ME, Al Othman MR, Al-Dosary SK. 2014. Phytochemical analysis and antimicrobial activity of medicinal plants against pathogenic microorganisms. Life Sciences Journal 11(7)

Bakr RO, El Raey MAE-A, Ashour RS. 2013. Phenolic content, radical scavenging activity and cytotoxicity of *Tamarix nilotica* (Ehrenb.) bunge growing in Egypt. Journal of Pharmacognosy and and Phytotherapy. 5, 12-20.

Bibi G, Haq IU, Ullah N, Mannan A, Mirza B. 2011. Antitumor, cytotoxic and antioxidant potential of *Aster thomsonii* extracts. African Journal of Pharmacy and Pharmacology. 5, 252–258.

Boulaaba M, Tsolmon S, Ksouri R, Han J, Kawada K, Smaoui A, *et al.* 2013. Anticancer effect of *Tamarix gallica* extracts on human colon cancer cells involves Erk1/2 and p38 action on G2/M cell cycle arrest. Cytotechnology 65(6), 927-936.

Creek DJ, Jankevics A, Burgess KE, Breitling R, Barret MP. 2012. IDEOM: an excel interface for analysis of LC-MS-based metabolomics data. Bioinformatics. 28(7), 1048-1049.

Drabu S, Chaturvedi S, Sharma M. 2012. *Tamarix gallica*-an overview. Asian Journal of Pharmaceutical and Clinical Research. 5, 17-19.

Fatima H, Khan K, Zia M, Rehman UT, Mirza B, Haq UI. 2015. Extraction optimization of medicinally important metabolites from *Datura innoxia* Mill: an in vitro biological and phytochemical investigation. BMC Complementary and Alternative Medicine. 15, 376.

Gul F, ShinwarI ZK, Afzal I. 2012. Screening of indigenous knowledge of herbal remedies for skin diseases among local communities of north west Punjab, Pakistan. Pakistan Journal of Botany. 44(5), 1609-1616.

Hamid I, Baharullah K, Sultan A, Ali R, Muhammad I, Muhammad NA, Hameed UR, Saqib W, Abdul W. 2012. Comparative efficacy of *Aloe vera* and *Tamarix aphylla* against cutaneous leishmaniasis. International Journal Basic Medical Sciences and Pharmacy 2, 42-45.

Haq IU, Mirza B, Kanwal S, Ahmad MS, Mirza B. 2012. Antioxidant/anticancer activities and phytochemical analysis of *Euphorbia wallichii* root extract and its fractions. Iranian Journal of Pharmaceutical Research. 11, 241–249.

Hassim N, Markom M, Anuar N, Dewi KH, Baharum SN, Mohd Noor N. 2015. Antioxidant and antibacterial assays on *Polygonum minus* extracts: different extraction methods. International Journal of Chemical Engineering. 2015, Article ID: 826709.

Keymanesh K, Hamedi J, Moradi S, Mohammadipanah F, Sardari S. 2009. Antibacterial, antifungal and toxicity of rare Iranian plants. International Journal of Pharmaceutics. 5, 82-85.

Khoshzaban F, Ghaffarifar F, Koohsari HRJ. 2014. *Peganum harmala* aqueous and ethanol extracts effects on lesions caused by Leishmania major (MRHO/IR/75/ER) in BALB/c Mice. Jundishapur Journal of Microbiology. 7(7).

Marasini BP, Baral P, Aryal P, Ghimire KR, Neupane S, Dahal N, *et al.* 2015. Evaluation of antibacterial activity of some traditionally used medicinal plants against human pathogenic bacteria. BioMed Research International. 2015, Article ID 265425

Marwat SK, Khan MA, Ahmad M, Zafar M, Rehman F, Sultana S. 2009. *Salvadora persica*, *Tamarix aphylla* and *Zizyphus mauritiana* three woody plant species mentioned in the Holy Qura'n and Ahadith and their ethnomedicinal uses in north-western part (D.I. Khan) of Pakistan. Pakistan Journal of Nutrition 8, 542-547.

Mohammedi Z, Atik F. 2011. Impact of solvent extraction type on total polyphenols content and biological activity from *Tamarix aphylla* (L.) karst. International Journal of Pharma and Bio Sciences. 2(1), 609-615.

Qadir MI, Abbas K, Hamayun R, Ali M. 2014. Analgesic, anti-inflammatory and anti-pyretic activities of aqueous ethanolic extract of *Tamarix aphylla* L. (Saltcedar) in mice. Pakistan Journal of Pharmaceutical Sciences 27(6), 1985-1988.

Qaiser M, Perveen A. 2004. Pollen flora of Pakistan-xxxvii, Tamaricaceae. Pakistan Journal of Botany. 36(1), 1-18.

Schatschneider S, Abdelrazig S, Safo L, Henstra AM, Millat T, Kim DH *et al.* 2018. Quantitative isotope-dilution high-resolution-mass-spectrometry analysis of multiple intracellular metabolites in *Clostridium autoethanogenum* with uniformly 13c-labeled standards derived from *Spirulina*. Analytical Chemistry 90(7), 4470-4477

Scheltema RA, Jankevics A, Jansen RC, Swertz MA, Breitling R. 2011. Peakml/mzmatch: a file format, java library, r library, and tool-chain for mass spectrometry data analysis. Analytical Chemistry. 83(7), 2786-2793.

Tatiya A, Tapadiya G, Kotecha S, Surana S. 2011. Effect of solvents on total phenolics, antioxidant and antimicrobial properties of *Bridelia retusa* Spreng stem bark. Indian Journal of Natural Products and Resources. 2, 442-447.

Tautenhahn R, Bottcher C, Neumann S. 2008: Highly sensitive feature detection for high resolution LC/MS. BMC Bioinfomatics. 9, 504.

Verma S, Singh SP. 2008. Current and future status of herbal medicines. Veterinary World. 1(11), 347-50.

Want EJ, Wilson ID, Gika H, Theodoridis G, Plumb RS, Shockcor J *et al.* 2010. Global metabolic profiling procedures for urine using UPLC-MS. Nature Protocols. 5(6), 1005-1018.

Zain M E, Awaad A S, Al-Outhman MR, El-Meligy R M. 2012. Antimicrobial activities of Saudi Arabian desert plants. Phytopharmacology 2(1),106-113.

AUTHOR CONTRIBUTIONS:

All experiments and biological assays carried out predominantly by A.H., and Z.G.K. I.U.H., P.A.D.W.. and N.F. advised on the methods. Manuscript written by A.H. and P.A.D.W..