

A close-up photograph of several ripe, red strawberries with green leaves, resting on a dark wooden surface. The strawberries are the central focus, with some in sharp focus and others slightly blurred in the background.

Journal of Medicinal Plant Research

Volume 9 Number 27, 17 July, 2015

ISSN 2009-9723



*Academic
Journals*

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Full Length Research Paper

A new pterocarpan from the leaves of *Abrus precatorius* L.

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Received 2 March, 2015; Accepted 15 July, 2015

A pterocarpan, 2,3,4,8-tetramethoxy-6a,11a-dihydro-6H-benzo[4,5]furo[3,2-c]chromene-7,9-diol (compound 1) was isolated from the dichloromethane extract of the leaves of *Abrus precatorius* L. The structure of the compound was elucidated by detailed spectroscopic analysis such as ¹H NMR, ¹³C NMR, distortionless enhancement by polarisation transfer (DEPT), heteronuclear multiple-quantum correlation (HMQC) and heteronuclear multiple-bond correlation (HMBC). The crude extracts displayed brine shrimp lethality and *in vitro* antimalarial activity. The methanolic crude extract demonstrated an A/B ratio of 9.7 signifying that it is more toxic against *Plasmodium falciparum* than brine shrimp.

Key words: Fabaceae, Papilionaceae, *Abrus precatorius*, pterocarpan, brine shrimp lethality, antiplasmodial activity.

INTRODUCTION

The plant *Abrus precatorius* L. (family: Fabaceae, sub-family: Papilionaceae) is a vine originally native to India that is now commonly distributed throughout tropical and subtropical regions of the world (Choi et al., 1989). Poisoning by the seeds of these species has been well documented (Morton, 1977, 1995; Anonymous, 1969; Saganuwan et al., 2011) with abrin, the glycoprotein responsible, being regarded as one of the most potent of all known toxins (Olsnes et al., 1974). Despite the known toxicity of *A. precatorius* seeds, its roots have been used since early 19th century as a substitute for licorice root, the source of the sweet oleanane-type triterpene glycoside, glycyrrhizin (Dymock et al., 1893; Hooper,

1894; Uphof, 2001; Prathyusha et al., 2010; Pokharkar et al., 2011; Attal et al., 2010). In *in vivo* studies, different organic extracts of the seeds induced antifertility activity (Abu et al., 2012), analgesic activity (Monago and Alumanah, 2005), antimicrobial activity (Bobbarala and Vadlapudi, 2009), hepatoprotective activity (Battu and Kumar, 2009), and can protect the kidney against alcohol-induced parenchymal injury (Ligha et al., 2009), while that of leaves induced analgesic activity (Nagaveni et al., 2012) and may be used in the management of asthma (Taur and Patil, 2012). The root decoction is used against vomiting, dysentery, uterine prolapse, epilepsy, stomachache, convulsions in children, conjunctivitis,

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asthma and as an aphrodisiac (Sujit, 2011; Janakiraman et al., 2012). The dried powdered leaves are crushed and the juice drunk against cough, fever and dizziness (Chhabra et al., 1990). The chemical composition (Mollik et al., 2009; Rajaram and Janardhanan, 1992) and nutritional potential (Rajaram and Janardhanan, 1992) of the seeds were investigated. The present study reports the isolation and structure elucidation by various spectroscopic techniques of a new pterocarpan, 2,3,4,8-tetramethoxy-6a,11a-dihydro-6H-benzo[4,5]furo[3,2c]chromene-7,9-diol (compound 1) from the dichloromethane extract of the leaves of *A. precatorius*. The crude extracts were evaluated for brine shrimp lethality and *in vitro* antiplasmodial activity.

MATERIALS AND METHODS

General experimental procedures

The melting point of the pure compound was determined on a Gallenkamp melting point apparatus (Sanyo, United Kingdom) with open capillary tubes and was uncorrected. The NMR spectra were obtained from Varian Gemini 200 MHz and a Bruker Avance DPX 300 MHz machine. MS analysis was performed on pure solid samples using the direct insertion probe (DIP) on a Fission Platform Mass Spectrometer operated at 70 eV and mass range set at 38-400 a.m.u. and using Atmospheric Pressure Chemical Ionization (APCI) on a Finnigan LCQ Deca machine, in either the positive mode (gives M+H) or negative mode (gives M-H as quasi molecular ion). Pre-coated plastic sheets (Polygram[®] sil G/UV₂₅₄) and aluminium sheets (Alugram[®] sil G/UV₂₅₄ of 20 cm by 20 cm Machery-Nagel GmbH and Co., KG) were used for Thin Layer Chromatography (TLC). Column chromatography was carried out using slurry packing with Kiesegel silica gel (0.063-0.2/70-230 mesh ASTM Kobian Kenya Ltd., Nairobi).

Plant

The leaves of the plant *A. precatorius* L. were collected in October 2000 based on information given by various herbalists on its use for the treatment of various diseases specifically malaria from the Mombasa County (latitude -4.05°, longitude 39.67°), Kenya. It was identified by Mr. Simon Mathenge, a taxonomist at the Botany Department, University of Nairobi and a voucher specimen (CNM/SM/09/01) deposited at the Nairobi University herbarium. The plant material was dried under shade and ground using a motor grinding machine.

Extraction and isolation

The air dried powdered leaves of *A. precatorius* (600 g) were extracted by maceration successively in hexane, dichloromethane and methanol (each 1200 ml, 3×48 h). The combined extracts of each solvent were concentrated under reduced pressure below 50°C to get the respective crude extracts. Dichloromethane crude extract of *A. precatorius* (2.98 g) was chromatographed over silica gel column (30 g, 100-200 mesh), eluted with gradients of EtOAc in *n*-hexane and gave 17 fractions (300 ml each). Fractions (14 to 17) obtained on elution with 100% EtOAc yielded compound 1 on repeated washing with diethyl ether and crystallization from MeOH with an *R*_f of value 0.70 (*n*-hexane-EtOAc, 50:50).

2,3,4,8-Tetramethoxy-6a,11a-dihydro-6H-benzo[4,5]furo[3,2-c]chromene-7,9-diol (compound 1)

Yield 25 mg (white crystals). m.p. found: 175.7-176.2°C; IR, 3400, 3299, 2900, 1576.2, 1306.7, 918.1, 872.7, 838.0, 730.0, 774.4 cm⁻¹; ¹H NMR spectral data (300 MHz, CDCl₃): δ 6.65 (1H, s, H-1), 6.61 (1H, s, H-10), 4.34 (1H, dd, J = 4.8, 10.8 Hz, H-6α), 3.55 (1H, m, H-6a), 3.68 (1H, t, J = 10.8, 10.9 Hz, H-6β), 5.41 (1H, s, 9-OH), 5.46 (1H, d, J = 6.9 Hz, H-11a), 5.63 (1H, s, 7-OH), 3.92 (3H, s, 4-OMe), 3.93 (3H, s, 3-OMe), 3.88 (3H, s, 2-OMe), 3.99 (3H, s, 8-OMe); ¹³C NMR Spectral data (75 MHz, CDCl₃): δ 148.1 (C-2), 144.2 (C-9), 144.1 (C-10a), 139.3 (C-8), 138.8 (C-7), 138.5 (C-4a), 138.1 (C-3), 137.3 (C-4), 123.0 (C-7a), 115.1 (C-1a), 105.1 (C-10), 104.1 (C-1), 78.5 (C-11a), 67.0 (C-6), 61.7 (8-OMe), 61.4 (4-OMe), 60.7 (3-OMe), 56.7 (2-OMe), 41.3 (C-6a); APCI (+ve mode, m/z (rel. int. %): 376 (88.6), 362 (20), 361 (100), 315 (9.3), 194 (31.4), 183 (12.1%), 181 (35), 179(36.4), 173 (18.9), 151 (10), 136 (21.4), 131 (16.4), 123 (14.3), 115 (30), 102 (17.9), 94 (12.1), 91 (27.1), 89 (19.3), 81 (11.4), 77 (27.1), 69(23.6), 65 (27.1), 63 (12.9), 53 (22.1), 51 (17.1), 43 (14.3), 39 (19.3).

Toxicity testing against the brine shrimp

Hatching shrimp

Brine shrimp eggs, *Artemia salina* Leach were hatched in artificial seawater prepared by dissolving 38 g of sea salt (Sigma chemicals Co., UK) in 1 L of distilled water. After 48 h incubation at room temperature (22 to 29°C), the larvae (nauplii) were attracted to one side of the vessel with a light source and collected with pipette. Nauplii were separated from eggs by aliquoting them three times in small beakers containing seawater.

Brine shrimp bioassay

The bioactivity of the extracts was monitored by the brine shrimp lethality test (Wanyoike et al., 2004). Samples were dissolved in dimethylsulphoxide (DMSO) and diluted with artificial sea salt water so that final concentration of DMSO did not exceed 0.05%. Fifty microliters of sea salt water was placed in all the wells of the 96-well microtiter plate. Fifty microliters of 4000 ppm of the plant extract was placed in row one and a two-fold dilution carried out down the column. The last row was left with sea salt water and DMSO only served as the drug free control. 100 µl of suspension of nauplii containing about 10 larvae was added into each well and incubated for 24 h. The plates were then examined under a microscope (12.5×) and the number of dead nauplii in each well was counted. 100 µl of methanol was then added and after 10 min, the total numbers of shrimp in each well were counted and recorded. Lethality concentration fifties (LC₅₀ values) for each assay were calculated by taking average of three experiments using a Finney Probit analysis program on an IBM computer (Wanyoike et al., 2004).

In vitro antimalarial test

Cultures of *P. falciparum*

Laboratory adapted *P. falciparum* cultures of the international reference isolates VI/S (chloroquine resistant) were used. The strains have been cultured and maintained at the Faculty of Pharmacy, University of Nairobi, Nairobi. The culture medium was a variation of that described by Wanyoike (2004) and consisted of

Table 1. Percent yield of extracts, brine shrimp toxicity and *in vitro* antiplasmodial activity of the extracts of the leaves of *A. precatorius*.

Extract	Hexane	Dichloromethane	Methanol	Emetine hydrochloride*	Chloroquine*
Percent yield of extract	0.70	0.67	1.00		
Brine shrimp toxicity: LC ₅₀ ±SD (µg/ml)	927.8±2.2	>1000.0	415.3±1.4	20.1±0.2	-
<i>In vitro</i> antiplasmodial activity: IC ₅₀ ±SD (µg/ml)	-	-	43.0±0.9	-	0.105±0.0

*Included as a positive control.

RPMI 1640 supplemented with 10% human serum, 25 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid and 25 mM NaHCO₃. Human type O+ erythrocytes (<28 days old) served as host cells and the cultures were incubated at 37°C in an atmosphere of 3% CO₂, 5% O₂ and 92% N₂.

Antiplasmodial bioassay

The *in vitro* semi-automated microdilution assay technique that measured the ability of the extracts to inhibit the incorporation of [³H]hypoxanthine into the malaria parasite was used (Wanyoike et al., 2004). For the test, 25 µl aliquots of culture medium were added to all the wells of a 96 well flat-bottom microculture plate (Costar Glass Works, Cambridge, UK). Aliquots (25 µl) of the test solutions were added, in duplicate, to the first wells, and a Titertek motorized hand diluter (Flow Laboratories, Uxbridge, UK) was used to make serial 2-fold dilutions of each sample over a 64-fold concentration range. Aliquots (200 µl) of a 1.5% v/v suspension of parasitized erythrocytes in culture medium (0.4% parasitemia; growth rate > 3-fold per 48 h) were added to all test wells. Parasitized and non-parasitized erythrocytes were incorporated into all tests. The plates were incubated at 37°C in a gas mixture 3% CO₂, 5% O₂ and 92% N₂. After 48 h, each well was pulsed with 25 µl of culture medium containing 0.5 µCi of [³H] hypoxanthine and the plates were incubated for a further 18 h. The contents of each well were then harvested onto glass fiber filters, washed thoroughly with distilled water, dried and the radioactivity in counts per minute (cpm) measured by liquid scintillation.

Computation of the concentration of drug causing 50% inhibition of [³H]hypoxanthine uptake (IC₅₀) was carried out by interpolation after logarithmic transformation of both concentration and cpm values using the formula:

$$IC_{50} = \text{antilog} \left[\frac{(\log X_1 + [(\log Y_{50} - \log Y_1) \times (\log X_2 - \log X_1)])}{\log Y_2 - \log Y_1} \right]$$

where Y_{50} is the cpm value midway between parasitized and non-parasitized control cultures and X_1 , Y_1 , X_2 and Y_2 are the concentrations and cpm values for the data points above and below the cpm midpoints (Wanyoike et al., 2004).

RESULTS AND DISCUSSION

The leaves of the plant *A. precatorius* were successively extracted with hexane, dichloromethane and methanol and the percent yield of each extract is as shown in Table 1. The brine shrimp toxicity and *in vitro* antiplasmodial

activity of the extracts of the leaves are also shown in Table 1.

Brine shrimp lethality and *in vitro* antiplasmodial test

The crude extracts (hexane, dichloromethane and methanol) of the leaves of *A. precatorius* plant were subjected to brine shrimp lethality test and their LC₅₀ values were determined. The *in vitro* antimalarial activities of the methanolic crude extract against chloroquine resistant VI/S strain of *P. falciparum* were also investigated and its IC₅₀ value determined. The methanolic crude extract of *A. precatorius* displayed a higher activity against brine shrimp (415.3 µg/ml). The hexane (927.8 µg/ml) and dichloromethane (>1000.00 µg/ml) extracts were about 2 times less active than the methanolic extract. The methanolic extract displayed an *in vitro* antiplasmodial activity of 43.0 µg/ml.

A/B ratio

When searching for new antimalarial agents, it is important to distinguish between specificity of action and non-selective toxicity. An estimation of therapeutic index in which the desired biological activity is compared with general toxicity is one in which selectivity of activity may be assessed (Abu et al., 2012). The brine shrimp lethality and activity against *P. falciparum* has been compared for the extracts and a ratio A/B for the two calculated (Monago and Alumanah, 2005). A value greater than one is considered indicative of more selective activity against *P. falciparum*, and a value less than one of more selectivity to brine shrimp.

The activities of the methanolic crude extract of *A. precatorius* against brine shrimp (A µg/ml) and *P. falciparum* (B µg/ml) *in vitro* was calculated to be 9.7. The A/B ratio of the methanolic extract of *A. precatorius* (9.7) demonstrates that it is more toxic against *P. falciparum* than brine shrimp.

Structure elucidation

Compound 1 was isolated from the dichloromethane

Table 2. ^1H and ^{13}C NMR data for compound 1.

Carbon	$\delta_{\text{C}}^{\text{c,d}}$	$\delta_{\text{H}}^{\text{b}}$	HMQC	HMBC
1	104.1 (d)	6.65 (1H, s)	6.65	C-11a
1a	115.1 (s)	-	-	C-6a
2	148.1 (s)	-	-	2-OMe
3	138.1 (s)	-	-	C-1
4	137.3 (s)	-	-	4-OMe
4a	138.5 (s)	-	-	C-1, C-11a, C-6
6	67.0 (t)	3.68 (1H, t, 10.8, 10.9) 4.34 (1H, dd, 4.8, 10.8)	3.68 4.34	C-11a
6a	41.3 (d)	3.55 (1H, m)	3.55	-
7a	123.0 (s)	-	-	7-OH, C-6, C-10, C-11a
7	138.8 (s)	5.63 (1H, s)	-	C-6a
8	139.3 (s)	-	-	8-OMe, 7-OH, 9-OH
9	144.2 (s)	5.41 (1H, s)	-	-
10	105.1 (d)	6.61 (1H, s)	6.61	9-OH
10a	144.1 (s)	-	-	C-6a, C-7, C-11a
11a	78.5 (d)	5.46 (1H, d, 6.9)	5.46	C-6, C-1
2-OMe	56.7 (q)	3.88 (3H, s)	3.88	-
3-OMe	60.7 (q)	3.93 (3H, s)	3.93	-
4-OMe	61.4 (q)	3.92 (3H, s)	3.92	-
8-OMe	61.7 (q)	3.99 (3H, s)	3.99	-

extract of the leaves of *A. precatorius* as white crystals. The IR spectrum showed peaks at 3299 and 3400 cm^{-1} (strong, broad hydroxyl groups), together with at 1307 cm^{-1} (C-O stretching), 2900 cm^{-1} (C-H stretching) and 1576 cm^{-1} (aromatic C=C stretching).

The ^1H NMR spectral data of compound 1 (Table 2) suggested a pterocarpan structure due to the splitting pattern of the protons at 5.46 (d, $J = 6.9$ Hz, H-11a), 4.34 (dd, $J = 4.8, 10.8$ Hz, H-6 α), 3.55 (m, H-6a) and 3.68 (t, $J = 10.8, 10.9$ Hz, H-6 β), related to the protons of the hetrocyclic ring B and the bridging protons of B and C rings, respectively (Tarus et al., 2002; Prathyusha et al., 2010). The chemical shifts appearing at $\delta 6.65$ and 6.61 were assigned to H-1 and H-10, respectively.

In addition, compound 1 possessed four methoxy groups, which appeared at $\delta 3.92$ (3H, s, 4-OMe), 3.93 (3H, s, 3-OMe), 3.88 (3H, s, 2-OMe) and 3.99 (3H, s, 8-OMe). These were placed on rings A and D. Two hydroxyl groups appearing at $\delta 5.41$ (s, 9-OH) and 5.63 (s, 7-OH) were assigned to the two OH groups attached to carbon 9 and 7, respectively.

A total of 19 signals were observed on ^{13}C NMR spectrum, while the Distortionless Enhancement by Polarisation Transfer (DEPT) showed a total of 1 methylene, 4 methyl and 4 methine groups (Table 2). Two methine protons were observed at the chiral centres

$\delta 5.46$ (1H, d, $J = 6.9$ Hz, H-11a) and at $\delta 3.55$ (1H, m, H-6a). The ^1H - ^1H COSY NMR spectrum showed coupling between protons $\delta 5.46$ (H-11a) and $\delta 3.55$ (H-6a). It also showed coupling between protons $\delta 4.34$ (H-6 α) and $\delta 3.68$ (H-6 β), $\delta 4.34$ (H-6 α) and $\delta 3.55$ (H-6a), $\delta 5.46$ (H-11a) and $\delta 3.55$ (H-6a).

The Heteronuclear Multiple-Quantum Correlation (HMQC) spectrum for compound 1 confirmed that the proton at $\delta 6.65$ (H-1) was attached to carbon at $\delta 104.1$ (C-1) while that at $\delta 5.46$ (H-11a) was attached to carbon at $\delta 78.5$ (C-11a). The proton at $\delta 3.55$ (H-6a) was attached to carbon at $\delta 41.3$ (C-6a), while that at $\delta 3.68$ (H-6 β) and 4.34 (H-6 α) were attached to carbon at $\delta 67.0$ (C-6). The methoxylated protons at $\delta 3.88, 3.93, 3.92$ and 3.99 were attached to carbons at 56.7 (C-2), 60.7 (C-3), 61.4 (C-4) and 61.7 (C-8), respectively. Heteronuclear Multiple-Bond Correlation (HMBC) assisted in the assignment of the quaternary carbons as shown in Table 2.

The mass spectrum of compound 1 showed a peak at $m/z 376$ (88.6%) corresponding to the chemical formula $\text{C}_{19}\text{H}_{20}\text{O}_8$. The suggested structure of compound 1 is 2,3,4,8-Tetramethoxy-6a,11a-dihydro-6H-benzo[4,5] furo [3,2-c]chromene-7,9-diol (Figure 1). It is a hitherto unknown natural product and has been isolated for the first time from the leaves of *A. precatorius*.

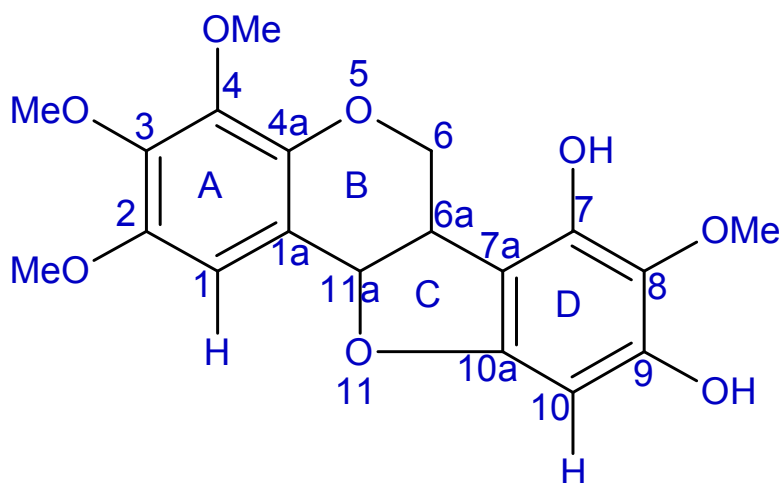


Figure 1. Structure of compound 1 2,3,4,8-Tetramethoxy-6a,11a-dihydro-6H-benzo[4,5]furo[3,2-c]chromene-7,9-diol.

Conclusion

A new pterocarpan (compound 1) from the dichloromethane extract of the leaves of *A. precatorius* has been isolated and reported for the first time. The methanol extract of the leaves showed *in vitro* antiplasmodial activity confirming the claim by the communities for the use of this plant in treating malaria.

ACKNOWLEDGEMENTS

The authors acknowledge WHO-MIM for the financial support and the Network for Analytical and Bioassay Services in Africa (NABSA) for recording NMR spectra.

Conflict of interest

The authors declare that they have no conflict of interest.

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Full Length Research Paper

Anti-acne activity of *Achillea* 'Moonshine' petroleum ether extract

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Received 6 March, 2015; Accepted 14 July, 2015

Achillea millefolium (yarrow) is a traditionally used plant to treat wounds. The present study was conducted to evaluate the anti-acne activity of *Achillea* 'Moonshine', a hybrid variety of *Achillea*. The plant was extracted in four solvents - petroleum ether, ethyl acetate, ethanol and water. These extracts were screened for anti-microbial, free radical scavenging, anti-tyrosinase, anti-inflammatory activity and cytotoxicity assays necessary to characterize its anti-acne activity. The most promising activity was determined in the petroleum ether extract. The minimum inhibitory concentration (MIC) value for the petroleum ether extract was 0.83 mg/ml against *Propioni bacterium acnes* and 0.37 mg/ml against *Staphylococcus*. The minimum bactericidal concentration (MBC) value for petroleum ether was 0.83 and 0.75 mg/ml for *P. acnes* and *Staphylococcus epidermidis*, respectively. Though the ethyl acetate had a high flavonoid and phenolic content it was observed that the IC₅₀ values for the petroleum ether extract for free radical scavenging activity was 64.81 µg/ml, which was higher than ethyl acetate. Petroleum ether also showed tyrosinase inhibition at 0.033 mg/ml. The extract was also able to decrease the inflammatory cytokines like TNF-α and IL-8, and showed no cytotoxicity against dermal fibroblasts. These results suggest presence of active anti-acne phytochemicals in the petroleum ether extract, making it a novel plant candidate for the treatment of acne.

Key words: Anti-acne, *Achillea* 'Moonshine', petroleum ether extract.

INTRODUCTION

Achillea millefolium Linn. commonly known as yarrow is a flowering plant from the Asteraceae family, and is represented by about 85 different species. It is widely found in Asia, North America and Europe (Li et al., 2011; Moradi et al., 2013). Traditionally, yarrow is purported to

be a diaphoretic, astringent, tonic, stimulant and mild aromatic (Benedek et al., 2007). The plant has a long history as a powerful 'healing herb', used topically for wounds, cuts and abrasions (Benedek et al., 2007). Previous studies have shown yarrow to have potential

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cosmetic uses (Chandler et al., 1982) including skin surface rejuvenation effects. However, the majority of the studies have been performed on the flowers of yarrow. Various species of *A. millefolium* used in garden traditions have been superseded with cultivars and hybrids with improved qualities. One such hybrid is *Achillea* 'Moonshine' which is a cross between *Achillea* 'Taygetea' and *Achillea clypeolata*. *Achillea* 'Taygetea' is an ornamental plant native to Europe while *Achillea clypeolata* is native to Balkan Peninsula. Both the plants have yellow colored flowers and are drought resistant. However, there has not been much research on individual species. Over and above, hybridization and intraspecific variability have complicated the taxonomy. As a result of this, most of the plants of genus '*Achillea*' are referred to as Yarrow and there is no clear identification of individual species. For the following study, the most popular *Achillea* that is used in the North American gardens *Achillea* 'Moonshine' was used.

Achillea 'Moonshine' is an herbaceous perennial plant, 1 to 2 ft high and blooms between June to September, and is resistant to drought and dry soil. It is known for its fern-like, aromatic, silvery to gray-green foliage and its tiny, long-lasting, bright lemon-yellow flowers which appear in dense, flattened, compound corymbs (to 2 to 3" across). The present research is aimed towards the study of anti-acne activity in the leaves and stem extracts of *Achillea* 'Moonshine'.

Acne vulgaris (cystic acne or simply acne) is a common human skin disease, occurring mostly during the onset of puberty. It is generally characterized by areas of skin with seborrhea, comedones, papules (pinheads), pustules (pimples), nodules and possibly scarring. The known causes for acne are infection by bacteria such as *Propionibacterium acnes* and *Staphylococcus epidermidis*, hormonal changes, genetic predisposition to acne, adverse effects of cosmetics, medications, or other factors. Hence, all scientific attempts in developing anti-acne drugs focus on addressing the above mentioned causes of acne (Toyoda and Morohashi, 2001).

Acne occurs due to blockage in the follicles resulting in the formation of comedones. The sebaceous glands become clogged with sebum and dead skin cells. Under these conditions, bacterium *Propionibacterium acne* can cause inflammatory lesions in the dermis around the comedones or microcomedo resulting in redness and scarring. The inhibition of *P. acnes* decreases the rupturing of comedones into the surroundings and helps to inhibit the progression of acne. Another organism which is implicated in the etiology of acne is a common skin micro flora, *Staphylococcus epidermidis* which promotes pustules and nodule formation. Thus, for screening anti-acne compounds the inhibition of both these organisms is of prime consideration in treating acne (Knor, 2005; Tanghetti, 2013).

Another factor that causes increased chances of acne is oxidative stress. The skin is exposed to oxidative

stress induced by reactive oxygen species (ROS) both endogenously and externally. ROS play an important role in production of inflammatory mediators such as interleukin-8 (IL-8) and tumor necrosis factor- α (TNF- α) by monocytes/macrophages during the progression of acne. Also, excessive and repeated stimulation by invading organisms such as *P. acnes* and *S. epidermidis* causes over production of ROS (Akamatsu and Horio, 1998). These ROS causes considerable damage to the tissues leading to increase in inflammatory responses. Thus, reduction of ROS by antioxidants and free radical scavengers as well as inhibition of these inflammatory mediators is necessary to prevent damage at the cellular and the tissue levels. Post inflammatory hyper pigmentation (PIH) characterized by pigmentation in dermis or epidermis is commonly seen in acne sufferers. It causes discoloration of skin following the inflammation caused during acne. Increase in pigmentation is due to alternations in the melanocytes, and thus, post-acne treatment requires clearing of affected skin area (Davis and Callender, 2010). A potential anti-acne candidate should thus also have an ability to clear the melanin depositions.

Many oral and topical antibiotics are used for the management and treatment of acne vulgaris. However, the prolonged use of these antibiotics has created a problem of antibiotic resistance among the acne inducing bacteria (Humphrey, 2012). Also, the long term use of topical agents causes skin irritation, leading to decrease in patient compliance and treatment failure. To overcome these drawbacks, natural products have been studied extensively as alternatives to complement the existing therapies for the treatment of acne because they provide a largely unexplored source of drug discovery and development.

In this study, the antibacterial activity of *Achillea* 'Moonshine' extracts against the two acne causing organisms (*P. acnes* and *Staphylococcus epidermidis*), free radical scavenging activity, anti-tyrosinase, anti-inflammatory potential as well as cytotoxicity against human skin cells to identify the most potent extract possessing anti-acne activity were determined.

METHODOLOGY

Collection of plant material

Eight full bloomed *Achillea* 'Moonshine' plants were collected from Holly Days Nursery Inc. near Philadelphia (United States) in June, 2013.

Preparation of extracts

The leaves and stems were washed three times with distilled water and grounded into fine powder using a simple grinder after they were air dried for 3 days. The plant material was then extracted with four solvents of increasing polarity (petroleum ether, ethyl acetate, ethanol and water) by maceration. The solvent was evaporated and the extracts were stored at 4°C till further use.

Determination of anti-microbial activity against *P. acnes* and *S. epidermidis*

Bacterial strains and media

The bacterial strains incorporated in this study were *P. acnes* (ATCC) and *S. epidermidis* (ATCC). *P. acnes* was incubated in brain-heart infusion media for 48 h at 37°C under anaerobic conditions in an anaerobic jar, while *S. epidermidis* was incubated in Muller Hinton broth for 24 h at 37°C, and their densities were adjusted to approximately 0.5 McFarland Standard.

Anti-microbial activity by disc diffusion method

Sterile 5 mm-Whatman No. 1 filter paper discs were used in the disc diffusion assay. The discs were soaked separately with 30 µl of each of the extract at a concentration of 6 mg/ml in dimethyl sulfoxide (DMSO) for organic solvents and sterile water for the water extract. Negative control discs were soaked in DMSO and distilled water for organic solvent and water extracts respectively. Erythromycin (60 µg/disc) was used as positive control. These discs were placed on Mueller-Hinton agar plates and brain heart infusion agar plates, previously swabbed with *S. epidermidis* and *P. acnes* respectively at a concentration of 10⁶ Colony Forming Units (CFU)/ml. The plates were incubated at 37°C for 24 h in case of *S. epidermidis* and 48 h anaerobically for *P. acnes*. Anti-bacterial activity was defined as the diameter (mm) of the clear inhibitory zone formed around the discs.

Minimum inhibitory concentration by tube dilution method

Tube dilution method was used to determine the minimum inhibitory concentration (MIC) against *P. acnes* and *S. epidermidis*. The extracts were dissolved in DMSO and were serially diluted from concentration 5 to 0.1 mg/ml in sterile culture tubes containing 0.2 ml sterile nutrient broth. The tubes were inoculated with 100 µl of bacterial suspension (approximately 10⁶ CFU/ml) in their respective broths and incubated at 37°C for 24 h in the case of *S. epidermidis* and 48 h anaerobically for *P. acnes*. After incubation, the tubes were examined for growth by visually observing the turbidity. MIC was defined as the minimum concentration that resulted in no growth of the bacteria. The test was carried out in triplicates (Chomnawang et al., 2005; Kim et al., 2008).

Minimum bactericidal concentration (MBC)

The MBC was determined by pipetting out 0.1 ml bacterial culture from MIC tubes which had no bacterial growth and plating it on to Muller Hinton agar for *S. epidermidis* and on Brain heart infusion agar plate in case of *P. acnes* for 24 h and 48 h respectively. After incubation, the concentration at which there was no single colony of bacteria was taken as MBC. The tests were carried out in triplicates.

Determination of total phenolic contents in the plant extracts

The concentration of phenolics in plant extracts was determined using Folin-Ciocalteu method (Pourmorad et al., 2006). The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract in concentration of 1mg/ml, 5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 4 ml 1M NaHCO₃. The absorbance was measured at 765 nm. The total phenolic content was calculated from a linear calibration curve with commercial gallic acid. The content of phenolics in extracts was expressed in terms of

gallic acid equivalent (µg of gallic acid/ml of extract). The experiments were carried out in triplicates.

Determination of total flavonoid content in the plant extracts

The content of flavonoids in the examined plant extracts was determined using spectrophotometric method (Pourmorad et al., 2006). Briefly, 0.5 ml of methanolic solution of the extract in the concentration of 1 mg/ml was mixed with 1.5 ml methanol, 0.1 ml of 10% AlCl₃ solution, 0.1 ml of 1M potassium acetate and 2.8 ml distilled water. The samples were incubated for 30 min at room temperature, and the absorbance was measured at 415 nm. The amount of flavonoid was calculated from a linear calibration curve with commercial quercetin. The flavonoid content was expressed in terms of quercetin equivalent (µg of quercetin/ml of extract). The experiments were carried out in triplicates.

Determination of free radical scavenging activity

The free radical scavenging activity of the extracts was determined on the ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals (Wu et al., 2009). The stock solution of extracts was prepared in methanol to achieve the concentration of 1 mg/ml. Dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62 µg/ml respectively. 0.1 ml from each dilution was mixed with 0.9 ml of methanolic solution of DPPH (0.4% w/v). After 30 min incubation in dark at room temperature, the absorbance was recorded at 517 nm. Control sample contained all the reagents except the extract. Percentage scavenging was calculated using equation:

$$\% \text{ Scavenging} = [\text{Ac} - \text{As}/\text{Ac}] \times 100$$

Where, Ac is absorbance of control (DPPH in methanol); As is the absorbance of the sample/extract. Extract concentration providing 50% inhibition (IC₅₀) was calculated from the graph of percentage scavenging against extract concentration. Tests were carried out in triplicate.

Determination of anti-tyrosinase activity

Tyrosinase inhibition activity in order to evaluate the ability of the extracts to reduce the post inflammatory hyper pigmentation was determined by spectrophotometric assay in a 96 well plate (Baurin et al., 2002). 70 µl of extract in DMSO was mixed with 30 µl mushroom tyrosinase (313 Units/ml). The mixture was incubated for 10 min at 37°C. L-tyrosinase (2 mM) in potassium phosphate buffer (50 mM, pH 6.5) was added and the resultant solution was again incubated for 20 min at 37°C. The final absorbance was read at 490 nm. Percentage inhibition was calculated by

$$\% \text{ Inhibition} = [\text{Ac} - \text{As}/\text{Ac}] \times 100$$

Where, Ac is absorbance of control; As is the absorbance of the sample. Extract concentration providing 50% inhibition (IC₅₀) was calculated from the graph of percentage inhibition against extract concentration. Tests were carried out in triplicate.

Cell line and culture media

Human monocytic THP-1 cells (ATCC TIB-202) were cultured in RPMI supplemented with heat inactivated fetal bovine serum while normal human dermal fibroblasts (Lonza CC2155) were cultured in FGM™-2 BulletKit™ (Lonza) and were incubated in a humidified

Table 1. Antimicrobial activity of various *Achillea* 'Moonshine' extracts by disc diffusion assay.

Extract	<i>P. acnes</i>	<i>S. epidermidis</i>
PE	13.6 mm±0.5	13.6 mm±0.5
EA	18 mm±1.73	15.3 mm±1.52
ET	17.1 mm±0.25	NA
Water	NA	NA
Erythromycin	23.8 mm±0.2	21.1 mm±1.25

Values indicate zone of inhibition in mm. Values expressed as mean ± SD (n=3). PE: Petroleum ether extract; EA: Ethyl acetate extract; ET: Ethanol extract; NA: No activity.

atmosphere with 5% CO₂ at 37°C.

Measurement of cytokine production

To determine the effect of the petroleum ether extract on production of pro inflammatory cytokines (TNF-α and IL-8), human monocytic THP-1 cells (10⁶ cells/ml) in a serum free media was stimulated with 100 µg/ml *P. acnes* (wet weight), alone or in combination of different concentrations (50 and 100 µg/ml) of the extract and were incubated for 18 h at 37°C. The culture supernatants were harvested. The concentrations of IL-8 and TNF-α in the supernatant were measured by ELISA (Kim et al., 2008).

Cytotoxicity assay

Normal human dermal fibroblasts (6000cells/well) were seeded in a 96 well plate and the extract treatment began 10 h after seeding. The general viability of the cultured cells was determined by XTT assay in which XTT is reduced to its formazan derivative. The cells were incubated with 50 and 100 µg/ml petroleum ether extract in DMSO for 18 h in a humidified atmosphere with 5% CO₂ at 37°C. 20µl of activating reagent was added to 1ml XTT and each well was treated with 50 µl of this activated XTT and was incubated for 5 h. The absorbance of the wells was measured at 475 nm and 660 nm. The tests were carried out in triplicates (Kim et al., 2008).

Data analysis

Data are expressed as mean ± standard deviation or as percentages for anti-microbial, free radical scavenging, total phenolic and flavonoid content determination and anti-tyrosinase activity. Statistical significance was determined by one way ANOVA using GraphPad Prism 6 software for free radical scavenging, anti-tyrosinase, anti-inflammatory activity and cell viability assay (Graphpad Software, Inc, La Jolla, California). Differences were considered significant at P value of less than 0.05.

RESULTS

Determination of anti-microbial activity

Disc diffusion assay

Disc diffusion assay was performed to determine the

Table 2. Antimicrobial activity against *P. acnes* and *S. epidermidis*.

Extract	<i>P. acnes</i>		<i>S. epidermidis</i>	
	MIC	MBC	MIC	MBC
PE	0.83	0.83	0.37	0.75
EA	1.25	1.25	0.53	1.06
ET	1.00	2.00	2.00	4.00

MIC- Minimum inhibitory concentration (mg/ml); MBC-Minimum bactericidal concentration (mg/ml), PE: Petroleum ether extract; EA: Ethyl acetate extract; ET: Ethanol extract.

antibacterial activity of the extracts against *P. acnes* and *S. epidermidis* (Table 1). The assay was performed as a pilot study to identify the active extracts. The water extract did not show any anti-microbial activity against both the organisms, and so was eliminated from further studies. The ethanolic extract did not show any activity against *S. epidermidis* but was active against *P. acnes*.

MIC determination by tube dilution

The anti-microbial activity of the extracts against the two acne causing organisms, *P. acnes* and *S. epidermidis* is shown in Table 2. All the extracts showed anti-microbial activity against the two acne causing organisms. The lowest MIC against *P. acnes* was observed in the petroleum ether extract (0.83 mg/ml) whereas that for *S. epidermidis* was also observed in the petroleum ether extract (0.37 mg/ml). MIC is the lowest concentration of the extract that will inhibit the visible growth of the bacteria while MBC is the lowest concentration of the plant extract that is required to kill the bacteria. As shown in the results, all the extracts show MBC value no more than four times the MIC values, and so all the extracts were considered to have antimicrobial properties. The results reported are performed in triplicates (French, 2006).

Total phenolic and flavonoid content

Total phenolic content was estimated by gallic acid (Table 3), and expressed as micrograms of gallic acid equivalent (GAE)/ml of extract. The highest phenolic content was determined in the ethyl acetate extract (360.3±0.082µg GAE/ml extract). The petroleum ether extract had the lowest amount of phenolic content. Total flavonoid content was estimated by quercetin (Table 3) and expressed as micrograms of quercetin equivalent (QE)/ml of extract. The highest flavonoid content was determined in the ethyl acetate extract (308±0.015 µg QE/ml extract). The petroleum ether extract had the lowest amount of flavonoid content.

Table 3. Total phenolic and flavonoid content in various extracts.

Extract	$\mu\text{g gallic acid/ ml extract}$	$\mu\text{g quercetin/ ml extract}$
PE	107.6 \pm 0.020	52 \pm 0.279
EA	360.3 \pm 0.082	308 \pm 0.015
ET	226.67 \pm 0.037	66.6 \pm 0.007

All experiments are performed in triplicates. Values expressed as mean \pm SD (n=3). PE: Petroleum ether extract; EA: Ethyl acetate extract; ET: Ethanol extract.

Table 4. % Free radical scavenging activity of various extracts.

Concentration ($\mu\text{g/ml}$)	BHT	PE	EA	ET
500	96.08 \pm 0.035	51.25 \pm 0.908	60.24 \pm 1.45	93.29 \pm 0.460
250	94.94 \pm 0.767	54.32 \pm 2.1	43.57 \pm 0.121	80.78 \pm 2.62
125	90.34 \pm 1.940	54.68 \pm 0.126	44.06 \pm 0.539	62.74 \pm 2.42
62.5	76.74 \pm 0.862	49.85 \pm 1.466	40.02 \pm 1.204	49.15 \pm 0.863
31.25	49.96 \pm 1.026	47.21 \pm 0.364	34.76 \pm 3.64	48.18 \pm 1.99
IC ₅₀	31.05	64.81	346.36	66.86

All experiments are performed in triplicates. Values expressed as mean \pm SD (n=3), PE: Petroleum ether extract; EA: Ethyl acetate extract; ET: Ethanol extract.

Table 5. % Tyrosinase inhibition of various extracts.

Concentration (mg/ml)	Benzoic acid	PE	EA	ET
0.67	98.82 \pm 0.22	89.54 \pm 1.68	85.41 \pm 0.64	98.69 \pm 0.19
0.34	98.86 \pm 0.29	90.28 \pm 0.47	75.86 \pm 0.67	98.18 \pm 0.43
0.17	97.99 \pm 0.99	76.35 \pm 0.89	59.03 \pm 0.68	77.27 \pm 3.72
0.09	93.70 \pm 0.44	65.81 \pm 0.91	57.75 \pm 0.24	65.76 \pm 0.24
0.04	78.16 \pm 4.36	58.29 \pm 0.387	52.80 \pm 1.79	52.45 \pm 0.17
0.02	48.1 \pm 0.017	34.18 \pm 0.060	33.51 \pm 0.008	10.54 \pm 0.019
IC ₅₀	0.021	0.033	0.037	0.038

All experiments are performed in triplicates. Values expressed as mean \pm SD (n=3), PE: Petroleum ether extract; EA: Ethyl acetate extract; ET: Ethanol extract.

Determination of free radical scavenging activity

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay was performed to evaluate the free radical scavenging activity. The % radical scavenging for all the three varieties is shown in Figure 1 and Table 4. DPPH is a stable free radical. On accepting hydrogen from a corresponding donor, its solutions lose the characteristic deep purple (λ_{max} 515–517 nm) color. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. The petroleum ether extract had the

highest free radical scavenging activity (IC₅₀: 64.81 $\mu\text{g/ml}$). BHT (butylated hydroxyl toluene; IC₅₀:31.05 $\mu\text{g/ml}$) was used as a reference compound. The extract concentrations have a significant level ($P < 0.05$) of free radical scavenging activity compared with the control.

Determination of anti-tyrosinase activity

The % inhibition of tyrosinase by the extracts is shown in Figure 2 and Table 5. The IC₅₀ value for benzoic acid, the reference compound, was 0.021 mg/ml. The petroleum

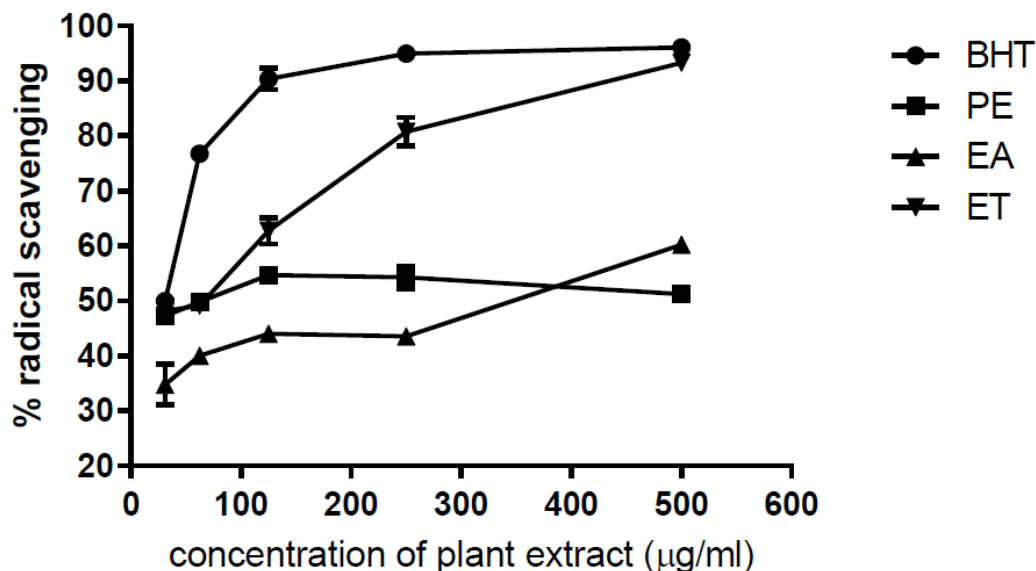


Figure 1. % radical scavenging activity of *Achillea* 'Moonshine' results expressed as the mean \pm SD (n=3), PE: Petroleum ether extract; EA: Ethyl acetate extract; ET: Ethanol extract.

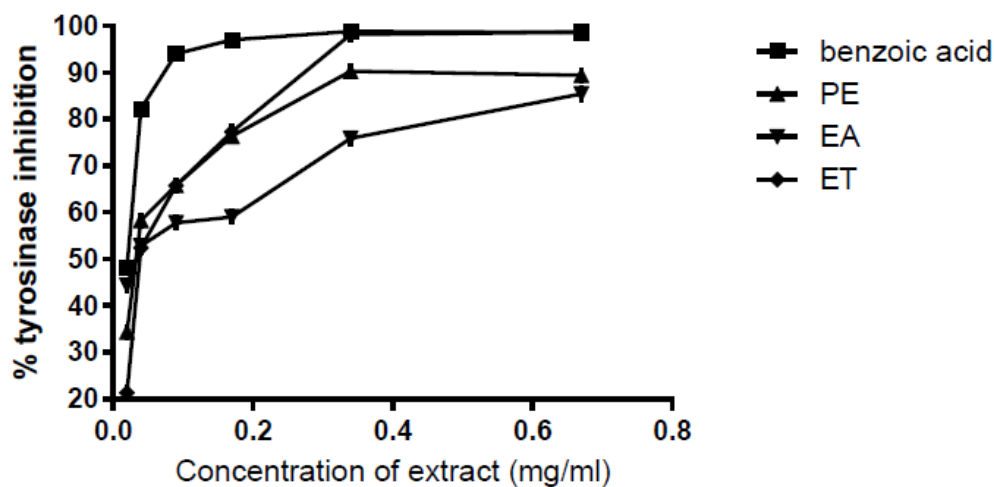


Figure 2. % tyrosinase inhibition activity of *Achillea* 'Moonshine'. Results expressed as the mean \pm SD (n=3)
PE: Petroleum ether extract; EA: Ethyl acetate extract; ET: Ethanol extract.

ether extract showed the lowest IC_{50} value for tyrosinase inhibition (IC_{50} : 0.033 mg/ml) compared to other extracts. The petroleum ether extract concentrations have a significant level ($P < 0.05$) of anti-tyrosinase activity compared with the control.

Measurement of cytokine production

The petroleum ether extract of *Achillea* 'Moonshine' was examined for its activity against inflammation induced by *P. acnes* in terms of inhibitory effects of cytokine

production. As shown in the Figure 3, cells treated with bacteria showed an increase in TNF- α and IL-8 production. However, co-cultures of cells with bacteria and extracts (50 μ g/ml) suppress the production of these cytokines. There was an elevated level of TNF- α cytokine production in 100 μ g/ml of extracts which was observed in some other studies as well (Kim et al., 2008).

Cytotoxicity assay

A potential candidate to be used in an anti-acne

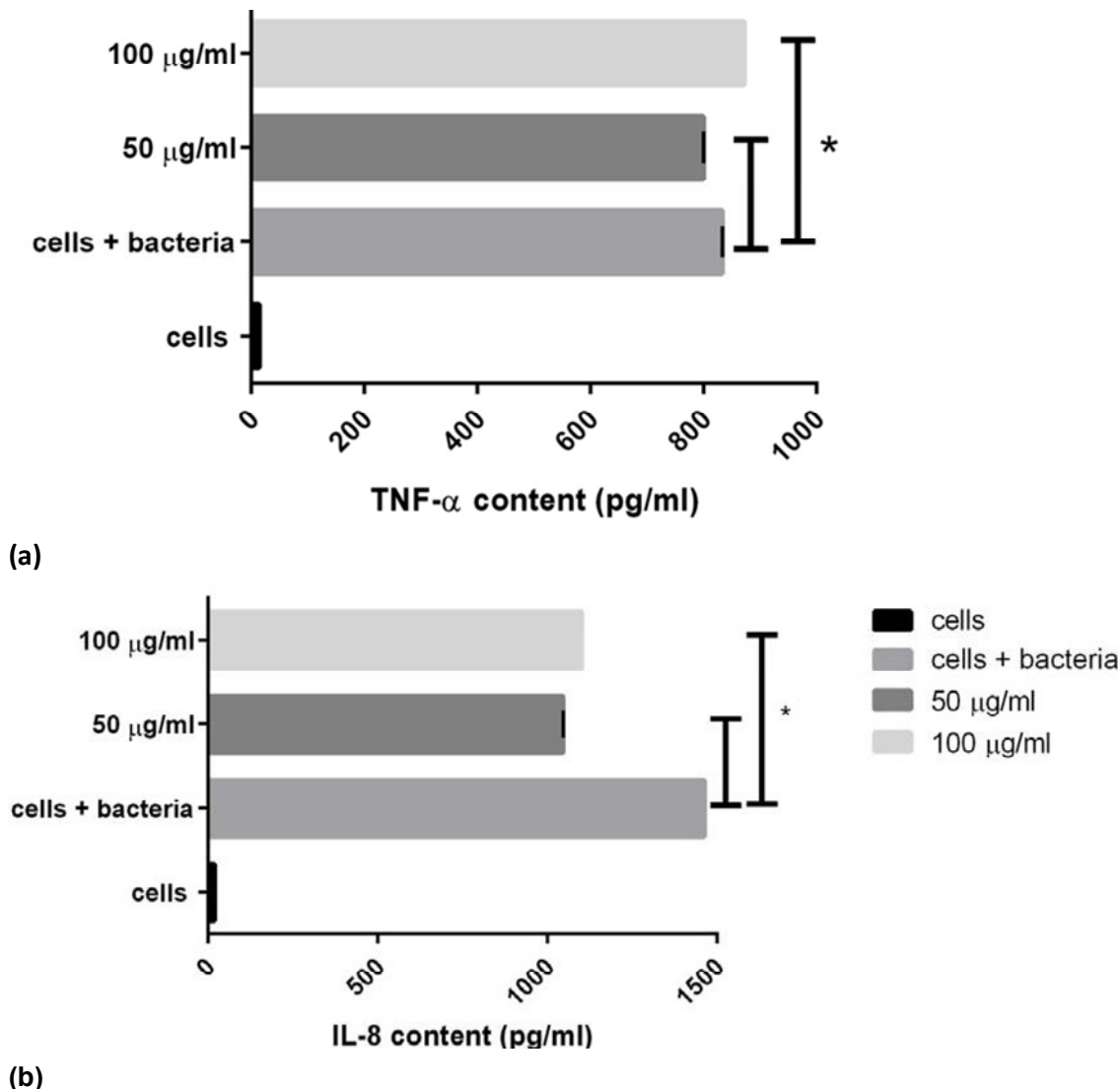


Figure 3. Inhibition of *P. acnes* induced pro-inflammatory mediators, TNF- α (top) and IL-8 (bottom) by petroleum ether extract. Data expressed as mean \pm SD. * $p < 0.05$ (n=3).

formulation should not be cytotoxic to human skin when applied topically. Figure 4 shows that the cell viability for human dermal fibroblasts is not significantly different for the cells grown with and without the plant extracts, suggesting no cytotoxicity.

DISCUSSION

The anti-acne screening presented in this study provides a plant candidate that is commonly grown in major parts of the world. To be qualified as plant with anti-acne properties, several different properties such as MIC and MBC against *P. acnes* as well *S. epidermidis* was performed. Other tests such as free radical scavenging, cytotoxicity test and anti-inflammatory tests were used to

determine the extract which had the most potential of reducing and curing acne. Some of the published anti-acne studies that are cited here have used plant material that is not easily available; hence our research provides data that gives insights to a unique property of yarrow that is known historically to treat wounds (Chandler et al., 1982). Anti-microbial activity assay, both by disc diffusion and tube dilution method for different extracts from *Achillea* ‘Moonshine’ showed that the petroleum ether extract had the highest potency against the main acne causing bacteria. Hence, the study is interested to know about the properties of petroleum ether extract in alleviating other causes and effects of acne.

The alcoholic fractions, usually rich in flavonoids have been used previously for anti-microbial treatments. For example, methanolic fraction from *Terminalia chebula*

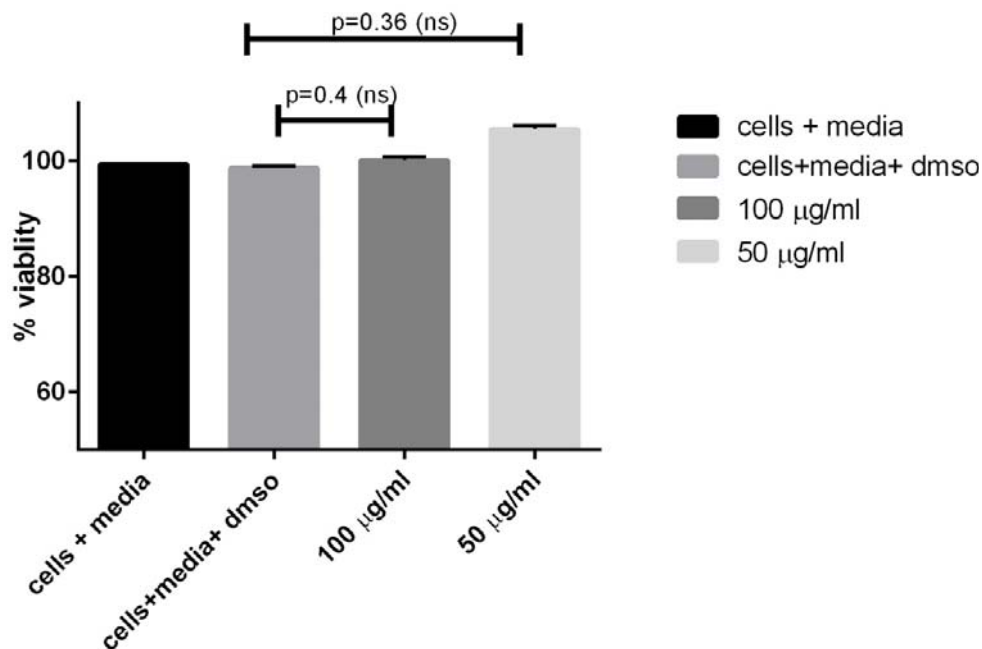


Figure 4. Cytotoxicity of petroleum ether extract against human dermal fibroblasts. Data expressed as mean \pm SD. * $p < 0.05$ (n=3); ns- non significant.

and *Terminalia bellarica* used in combination showed more anti-microbial activity against several microbes including *P. acnes* and *S. epidermidis* (Greeshma et al., 2006). Batubara et al. (2009) studies on anti-acne potency in Indonesian medicinal plants also showed higher anti-microbial activity in the alcohol fractions. However, this study report that the petroleum ether extracts from *Achillea* showed a high potency against acne causing organisms. Petroleum ether extracts from medicinal plants have been shown to contain majorly alkaloids and tannins (Ghumare et al., 2014). Tannins have natural astringent properties, and have been used topically to treat acne (Bedi et al., 2002). Alkaloids such as achilleine isolated from *A. millefolium L.* have been used as homeostatic and could be possibly one of the compounds in the petroleum ether extract contributing to the anti-acne effects (Miller et al., 1954).

This study also reported free radical scavenging activity more in the petroleum ether extract (Table 4). Generally, the free radical scavenging activity is attributed to flavonoids and phenolic compounds which are high in ethyl acetate extracts (Table 3). But, in this study the petroleum ether fraction had the highest radical scavenging activity, which indicates presence of certain potent non polar compounds such as fatty acids, certain flavones and steroids with high radical scavenging potential. Free radical scavenging activity was high in ethyl acetate extracts but this extract did not show high potency in killing *P. acnes* and *S. epidermidis* compared to petroleum ether extracts which is one of the most important factors in anti-acne treatment.

The tyrosinase inhibition activity was significant in the petroleum ether extract compared to the control. Compounds such as fatty acids and steroids easily solubilize in petroleum ether. These non-polar phytochemicals act as tyrosinase inhibitors by either competitively inhibiting the enzyme or by chelating the copper ion at the catalytic site of the enzyme. For some lipids, the inhibitory action is proposed due to binding of the compound to some site of the tyrosinase, except the catalytic site. Polyphenols are also considered to be potent tyrosinase inhibitors, but in this case, these compounds are not as effective as the non-polar phytochemicals because the petroleum ether extract which generally contains non polar compounds was more effective than the other extracts in which the polyphenols are soluble.

Petroleum ether extracts also showed anti-inflammatory effects that contribute in the properties of *A. 'Moonshine'* to qualify as a treatment for acne. The cytotoxicity tests on human dermal fibroblasts also showed *Achillea* to be safe for topical use. Based on the different anti-acne screens the petroleum ether extracts demonstrated to have the best potential as an anti-acne agent.

CONCLUSION AND RECOMMENDATION

Based on the current findings, the petroleum ether extract of *Achillea 'Moonshine'* showed the most promising anti-acne activity. It had potent anti-microbial, free radical

scavenging, anti-tyrosinase and anti-inflammatory activity. The extracts were also nontoxic to the dermal fibroblasts making it a good candidate for topical application. The plant is easily available in North America, Asia and Europe. These results and the increased availability of *Achillea* 'Moonshine' makes it an ideal novel plant candidate for the treatment of acne. Further studies need to be focused on isolation and characterization of the phytoconstituents responsible for anti-acne activity.

ACKNOWLEDGEMENTS

Research was funded by the Startup grant given to Dr. Bela Peethambaran by University of the Sciences, Philadelphia.

Conflicts of interest

The authors have none to declare.

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