Elucidating the Biological Activity of *Salvadora persica* and *Salvadora oleoides* by Exploiting *In silico* and Wet Lab Approaches

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2015

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Dedicated To Beloved Ammi Abbu

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In the name of ALLAH, the most beneficial and the most merciful. I am thankful to Allah almighty for the precious gift of life.

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List of abbreviations

AST	Serum aspartate aminotransferase
CIDS	Cholistan institute of desert studies
DEN-2	Dengue virus type-2
DMEM	Dulbecco's Modified Eagle Media
DPPH	2, 2-di-phenyl-2-picryl hydrazyl hydrate
FBS	Fetal Bovine Serum
FDA	Food and drug administration
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human Immunodeficiency virus
HSV	Herpes Simplex virus
MTT	Methylthiazoletetrazolium
PBS	Phosphate Buffered Saline
ROS	Reactive oxygen species
SARS	Severe acute respiratory syndrome
VHSV	Viral hemorrhagic septicemia virus
VSV	Vesicular stomatitis virus
WHO	World health organization

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Abstract

Natural products like plant extracts and compounds are source of lead molecules used as drugs. Medicinal plants have been used to cure various diseases. 25% of the modern medicines are derived from plants and about the same percentage is the synthetic analogue of phytochemicals. Low cost and less toxicity of plant derived compounds make them very important candidates to be used as potential drugs. In this study two plants *Salvadora persica* and *Salvadora oleiodes* of Pakistan origin have been evaluated for their biological activity by using *in silico* and wet lab approaches. Current study investigated the bactericidal effects of these plants and obtained results indicated potent efficacy against multi drug resistant strains of *Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella typhi*. Roots and leaves extracts of these plants shown good free radical scavenging capability in DPPH assay. *In vitro* cytotoxicity evaluation using MTT assay showed that these extracts are non-toxic even at high concentrations. Different compounds of these plants were able to interact and bind to various bacterial multi drug resistance causing proteins and HCV proteins *in silico*. Among these phytochemicals a compound, Salvadorin, shown most stable binding with target proteins. After analyzing data and results depicted in this study, it is recommended to purify and characterize these plant extracts by using comprehensive techniques and evaluate their activities specifically, which might help in discovering new drugs with broad therapeutic potentials.

Chapter 1

Introduction

Medicinal plants have been used to cure various diseases. Natural products like plant extracts and compounds are source of lead molecules used as drugs. 25% of the modern medicines are derived from plants and about the same percentage is the synthetic analogue of phytochemicals (Kunle *et al.,* 2012). Plants e.g. Ginseng, Ginkgo, Saw palmetto and Ispaghol are popular for the treatment of several disorders (Alzaher, 2014). Antimicrobial, anti-cancer, anti-diabetic and many other biological activities of plants have been reported. Published data about the medicinal plants is increasing every day.

Many medicinal plants have anti-viral potential. Antiviral activities of plants against Human Immunodeficiency virus (HIV), Herpes Simplex Virus (HSV), Human influenza virus, Hepatitis B virus (HBV), Hepatitis (HCV), pox virus and Severe Acute Respiratory syndrome (SARS) virus have been reported (Liu & Du, 2012). Some plant extracts have shown anti-viral effects against the strains which are resistant to conventional antiviral agents (Rajasekaran *et al.,* 2014). Number of phytochemiclas and crude extracts having anti-HCV potential have been identified. Plants reported for anti-HCV potential include *Acacia nilotica* L., *Embelia schimperi*, *Boswellia carterii*, *Trachyspermum ammi* L., *Piper cubeba* L., *Q. infectoria, Quercus infectoria*, and *Syzygium aromaticum* (Hussein *et al.,* 2000).

World health organization (WHO) is aiming to include medicinal plants into healthcare in the countries where use of plants for medical purposes is practiced (Nikam *et al.,* 2012). A beneficial use of plants can be in the production of protein based therapies and vaccines because several suggest that pharmaceutical grade proteins are developed

from plant sources. Several reports suggest the use of crude plant extracts as remedy as most of the plants have low cytotoxicity.

In this study two plants *Salvadora persica* and *Salvadora oleiodes* of Pakistan origin have been evaluated for their anti-HCV activity. *Salvadora* belongs to *Salvadraeceae* family.

Salvadora oleiodes, a bushy tree found in Pakistan and India, is reported for anti-oxidant, anti-inflammatory and anti-microbial activities (Arora *et al.,* 2014).

Salvadora persica is the popular medicinal plant throughout the Indian subcontinent and Muslim world commonly known as “Miswak”. WHO has promoted it for oral hygiene. *S. persica* is traditionally used in the treatment of leprosy, ulcers, gonorrhea, rheumatism, scurvy, dental diseases and tumors (Kumar *et al.,* 2012). Many studies prove that extract of *Salvadora Persica* has many biological characteristics including antibacterial, antifungal, antiplaque effect, anticaries, anti–inflammatory, and reduces gingivitis and gingival bleeding. It contains a large number of medicinal compounds for example salvadoricine, salvadourea, di-benzyl thiourea, rutin, trimethyl amine, thioglucoside, potash, sulphur, chlorine etc. Benzyl isothiocynate is reported to be responsible for antibacterial and anti-viral activities. Anti-viral activities of *S. persica* extracts have also been reported. (Kumar *et al.,* 2012; Sofrata *et al.,* 2012; Taha, 2008).

Broad range of biologically active compounds and range of reported biological activities of *S. persica* and *S. oleoides* extracts make them strong candidate as medicinal plants to be tested against more viruses as it has only been studied against Herpes Simplex virus (HSV).

Aims and Objectives

The objectives of the study included:

- To evaluate the biological activity of *S. persica* and *S. oleiodes* extracts.
- To determine and analyze the active antiviral and bactericidal compounds in *S. persica* and *S. oleiodes* extracts.

Chapter 2

Literature review

2.1. Medicinal plants

Medicinal plants have been used to cure various diseases since antiquity. Man has struggled through pain and got awareness about medicinal plants. Action of medicinal plants has been acknowledged by the science and they are included in modern pharmacotherapy (Efferth & Greten, 2014). According to WHO, 80% of the world's population in developing countries relies on herbal medicines (Kumar, 2014). 25% of the modern medicines are derived from plants and about the same percentage is the synthetic analogue of phytochemicals (Kunle *et al.,* 2012). The knowledge of medicinal plants, therefore, has benefitted the pharmacists and physicians to combat the challenges of health of man. Pakistan is blessed with unique floral diversity and different climatic zones.

WHO has planned a strategy to promote and development of traditional medicine, which includes identification of medicinal plants, developing cooperation between traditional and modern pharmacists and cultivation of herbs to prevent their destruction (Naseri, 2004). Plants e.g. Ginseng, Ginkgo, Saw palmetto and Ispaghol are popular for the treatment of several disorders (Alzaher, 2014). Published data about the medicinal plants is increasing every day.

2.2. Medicinal plants and their activities

Chronic disorders show the increased levels of free radicals and (ROS) which are highly reactive and can alter cellular constituents (Alfadda, & Sallam, 2012). Plants are natural reservoirs of antioxidants and they are proved to have significant antioxidant activities.

We can count many biological activities of medicinal plants cited in the literature. Some of these activities are listed as under:

Antimicrobial (Vashist & Jindal, 2012), Anti-cancer (Sakarkar & Deshmukh, 2011), Anti-diabetic (Patel *et al.,* 2012), Anti-atherosclerosis (Ismail *et al.,* 2012), Immunomodulatory (Akram *et al.,* 2014), Reno-protection (Musabayane, 2012), Hepatoprotection, (Kumar *et al.,* 2011).

To study the effects of medicinal plants against various diseases many animal models have been employed. Animal models for Diabetes, Autoimmune encephalitis, Bowel disease, Hyperlipidemia, Arthritis, Hepatic and renal toxicity, Cataract and many viral diseases etc. can be pointed in the literature (Rafieian-Kopaei, 2011).

2.3. Medicinal plants in viro therapy

Medicinal plants having anti-viral potential against many viruses have been reported including HIV, HSV, Human influenza virus, HBV, HCV, pox virus and SARS virus (Liu & Du, 2012). Some plant extracts have shown anti-viral effects against the strains which are resistant to conventional antiviral agents (Rajasekaran *et al.,* 2014).

Mechanisms involved in anti-viral activity may vary among different viruses. Some plant extracts show immunostimulatory functions e.g. *Heracleum maximum* stimulated interlukin-6 confirming its immunostimulatory function (Verma *et al.,* 2015). Besides immunostimulatory function some plants may contain phytochemicals which show antiviral properties e.g. Pandanin which is a lectin isolated from *Pandanus amaryllifolius* had antiviral effect against HSV (Dhawan, 2012). Some crude extracts of some other plants have also shown antiviral properties.

Table 2.1 Reported plants and their anti-viral activities

Virus	Medicinal plant	Reference
Human immunodeficiency virus (HIV)	*Nostoc ellipsosporum* (cyanobacteria)	Gustafson *et al.,* 1997
	Bridelia micrantha	Bessong *et al.,* 2006
Herpes simplex virus (HSV)	*Podophyllum peltatum*	Bedows and Hatfield, 1982
	Phyllanthus urinaria	Yang *et al.,* 2007
	Carissa edulis	Tolo *et al.,* 2006
	Apocynaceae	Tolo *et al.,* 2006
Influenza virus	*Geranium sanguineum* L.	Pantev *et al.,* 2006
	Elderberry extract	Zakey-Rones *et al.,* 2004
Viral hepatitis (A, B, C)	*Phyllanthus* species *P. amarus* (L.), *P. niruri* (L.) *P. urinaria*	Wang *et al.,* 1995
	Ardisia chinensis	Leung *et al.,* 2006
	Pithecellobium clypearia	Leung *et al.,* 2006
	Oenanthe javanica	Wang *et al.,* 2005
	Acacia nilotica L.	Rehman *et al.,* 2011
	Boswellia carterii	Hussein *et al.,* 2000
	Embelia schimperi	Hussein *et al.,* 2000
	Trachyspermum ammi	Hussein *et al.,* 2000
	Syzygium aromaticum	Hussein *et al.,* 2000
	Q. infectoria	Hussein *et al.,* 2000
	Piper cubeba	Hussein *et al.,* 2000
	Boehmeria nivea L.	Huang *et al.,* 2006
	Polygonum cuspidatum Sieb	Chang *et al.,* 2005

	Saxifraga melanocentra Engl	Zuo *et al.,* 2005
Severe acute respiratory syndrome (SARS) virus	*Glycyrrhiza uralensis* Fisch	Li *et al.,* 2005
	Lycoris radiate	Li *et al.,* 2005
Human rotaviruses (HRV)	*Stevia rebaudiana*	Takahashi *et al.,* 2001
	Haemanthus albiflos	Husson *et al.,* 1994
Polio virus	*Guazuma ulmifolia* Lam.	Felipe *et al.,* 2006
Viral hemorrhagic septicemia virus (VHSV)	*Olea europaea* L.	Micol *et al.,* 2005
Vesicular stomatitis virus (VSV)	*Trichilia glabra* L.	Cella *et al.,* 2003
Human adenovirus type 1	Black soybean extract	Yamai *et al.,* 2003
Dengue virus type-2 (DEN-2)	*Azadirachta indica* Juss. (Neem)	Parida *et al.,* 2002

WHO is aiming to include medicinal plants into healthcare in the countries where use of plants for medical purposes is practiced (Nikam *et al.,* 2012). These countries should sponsor research programs aimed to discover modern drugs from phytochemicals. Improved separation technologies and analysis techniques should be practiced to screen anti-infectious agents from plants.

A beneficial use of plants can be in the production of protein based therapies and vaccines because several suggest that pharmaceutical grade proteins are developed from plant sources. We have the biggest example of the expression of HBV surface antigen in plants for the production of subunit vaccine and its successful testing in animals and humans (Guan *et al.,* 2014). Efforts are being made to express virus like particles in plants (Thuenemann *et al.,* 2013).

Several reports suggest the use of crude plant extracts as remedy as most of the plants have low cytotoxicity. But this kind of approach cannot get approval from organization like food and drug administration (FDA) of America. So, there should be an aspect of isolation, purification and characterization of active product from the plants. Moreover, various studies have also shown hepatotoxicity caused by these plants extracts in the crude form (Fakurazi *et al.,* 2012). So, strategies should be made to address these issues as well.

2.4. Medicinal plants against Hepatitis C Virus

HCV is a serious problem of world. Some Phytochemicals e.g. polystrols, phytosterols, silymarin, glycyrrhizin, catechin, vitamin E and N-acetylcysteine have also been reviewed for their anti-viral potentials (Jassim & Naji, 2003; Patrick, 1999).

Herbs like Silybin and Oxymatrine have anti-HCV activity. Their use reduced the serum aspartate aminotransferase (AST) levels and resulted in HCV clearance (Liu *et al.,* 2003).

Chinese herbals like Bing Gan Tang, Yi Zhu decoction, and Yi Er Gan Tang showed anti-HCV effects used alone or in combination.

Methanolic and aqueous extracts of plants like *Acacia nilotica* L., Willd ex Delile, *Boswellia carterii, Embelia schimperi, Quercus infectoria*, *Trachyspermum ammi* L., *Piper cubeba* L., *Q. infectoria, Syzygium aromaticum* L., *Acacia nilotica, Viscum album, Agaricus blazel, Mori cortex radicus* showed anti-HCV potentials (Hussein *et al*., 2000; Rehman *et al.,* 2011).

Therefore, phytochemicals can be marked for further analysis and development for discovery of effective antiviral therapy. Some compounds can even be modified to enhance their activities.

2.5. *Salvadora persica (S. persica)*

Salvadora belongs to salvadraeceae family which has two species i.e. *S. persica* and *S. oleoides* in Indian subcontinent along with a new specie described from Sindh, Pakistan named as *S. alii* (Tahir *et al.,* 2010).

Name:	Salvadora persica
Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Brassicales
Family:	Salvadoraceae
Genus:	Salvadora
Species:	persica

S. persica is popular among the Muslim world and India for its use as chewing stick which is commonly termed as “miswak”. WHO has promoted it for oral hygiene. It is used to cure diseases like cough, asthma, bronchitis and scurvy as natural remedy. It has antibacterial and anti-inflammatory activities (Khalessi *et al.,* 2004).

S. persica is a small shrub like deep rooted tree. Its leaves are ovulate and fruit commonly named as “pilu” are pink or purple-red colored (Katewa *et al.,* 2004). *S. persica* is distributed variably from Algeria, Egypt to Pakistan, India, and Sri Lanka, and to Uganda and Zimbabwe (Alali & Al-Lafi, 2003).

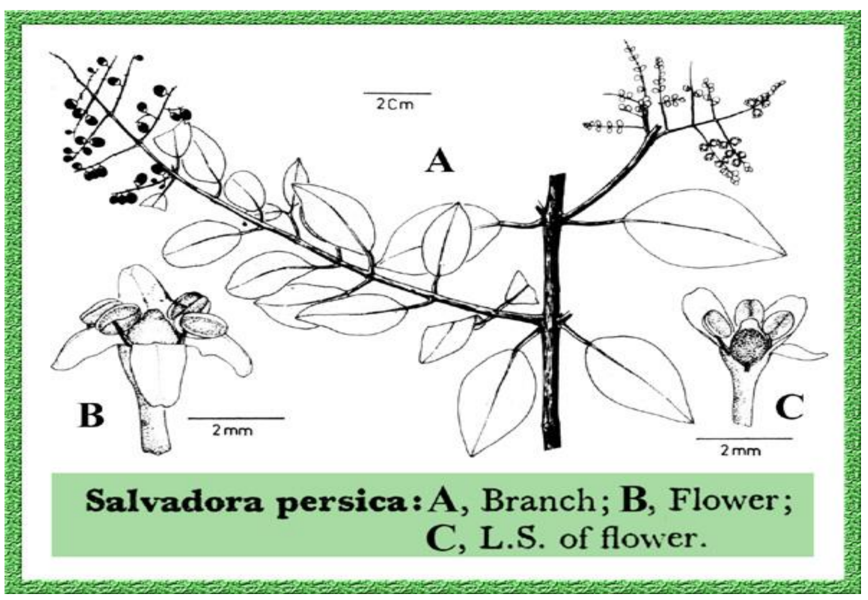


Figure 2.1: Illustration present in flora of Pakistan (eFloras of Pakistan)

2.5.1. Phytochemicals

Almost every part of this plant is rich in phytochemicals including biological active chemicals i.e. alkaloids, oils, flavonoids, steroids, carbohydrates, saponins and terpenoids. Its roots, leaves and stem are rich in trimethylamine, an alkaloid (Kumar *et al.,* 2012).

Table 2.2 Phytochemicals present in different parts of *S. persica*

Seed	Oil containing lauric acid, myristic and palmitic acid
Root	Benzyl glucisinolate, m-anisic acid, gammamonoclinic Sulphur, sitosterol
Stem	Octacosanol, β-sitosterol, triacantanol, benzylamide derivatives

A compound named benzyl isothiocyanate derived from root showed anti-HSV I activity (Naeini *et al.,* 2014).

2.5.2 Medicinal uses

Crude extracts of stems, roots and leaves are reported for treatment of various diseases. Primarily used as oral hygiene as tooth brush. It is used for the treatment of diseases like cough, asthma and fever. This plant is reported for anti-ulcer, anti-viral, anti-bacterial and anti-fungal activities (Naeini *et al.,* 2014).

S. persica contains compounds which remove plaque from teeth and have antibacterial activities (Almas, 2002). Several aqueous and alcoholic extracts showed anti-bacterial activities against Streptococcus mutans, Haemophilus influenzae, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, and Lactobacillus acidophilus (Ali *et al.,* 2002; Sofrata *et al.,* 2008).

A study claimed its anti-ulcer activity and its protective action against ulcer (Monforte *et al.,* 2001). Moreover, its anti-covulsant and analgesic properties are also been reported studied on mice (Monforte *et al.,* 2002; Sulaiman *et al.,* 1996). Fresh miswak had no cytotoxic effects (Patel *et al.,* 2012).

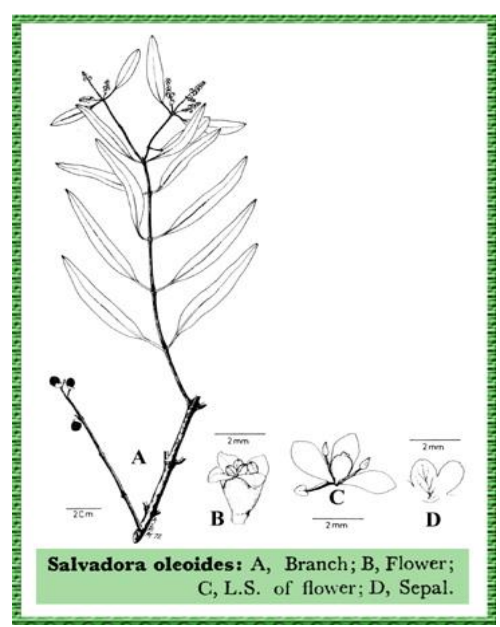
A study on mice showed that presence of its stem in diet lowered cholesterol and fats in mice.

S. persica is one of the plants that are cited in the Holy Quran (Ahmad *et al.,* 2009). It helps to maintain the oral hygiene and general health. Large number of reported medicinal applications suggest the development of modern drugs from this plant after investigating its bioactivity, mode of action, and pharmacotherapeutics on scientific lines. Moreover, modern biotechnological approaches have also not been tried on this plant to manipulate rapid biosynthesis of target metabolites.

2.6. ***Salvadora oleoides (S. oleoides)***

S. oleoides like *S. persica* is a xerophyte and belongs to the same family as that of *S. persica.* It is a small shrub like tree which differs in some aspects from *S. persica*. Its leaves are lanceolate and fruit is yellow in color which is also termed as “pilu” (Arora *et al.,* 2014). It is commonly found in Indian sub-continent, tropical Africa, Egypt and China. In Pakistan, it is present in enormous numbers in graveyards of south Punjab (Yadav *et al.,* 2008).

Name:	Salvadora oleoides
Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Brassicales
Family:	Salvadoraceae
Genus:	Salvadora
Species:	oleoides



Salvadora oleoides: A, Branch; B, Flower; C, L.S. of flower; D, Sepal.

Figure 2.2: Illustration present in flora of Pakistan (eFloras of Pakistan)

2.6.1. Photochemistry

Alkaloids, carbohydrates, steroids, tannins, saponins, glycosides, fats and oils are present in extracts of leaves and stem. Seeds are rich in oils and fruits are good source of sugars and calcium. Seeds contain quercetin and rutin which are flavonoids. Saturated and unsaturated fats are also present. A dihydroisocoumarin have also been isolated from *S. oleoides*. Novel polyamides are also isolated from its methanolic extracts (Garg *et al.,* 2014).

2.6.2. Medicinal uses

A large number of medicinal uses have been reported for *S. oleoides*. Its leaves are reported for their use in the treatment of enlarged spleen, rheumatism, piles, asthma and fever. It is used as purgative in horses (Upadhyay *et al.,* 2010).

Leaf extracts are used to treat inflammation and abdominal pain. Anti-inflammatory, anti-ulcer and analgesic properties are also been cited (Ahmad, 2007).

Ethanolic extracts of *S. oleoides* reduced blood glucose levels. It showed hypoglycemic and hypolipidemic activities and showed an overall beneficial antidiabetic potential

(Yadav *et al.,* 2008). Crude extracts also showed anti-oxidant activity (Dhankhar *et al.,* 2012).

Benzene, methanolic and aqueous extracts are reported for their antimicrobial potential compared with streptomycin. Different extracts of *S. oleoides* are employed against *E. coli, S. aureus, Staphylococcus epidermidis, Proteus vulgaris*, *Proteus mirabilis, Klebsiella pneumonia, Bacillus subtilis*, *Bacillus cereus,* and some fungi (*Aspergillus niger, Aspergillus fumigatus* and *Candida albican*) to confirm their anti-microbial potential (Kumar *et al.,* 2012).

Alcohol and water extracts showed anti-inflammatory effects in different animal models against standard drug Indomethacin.

Polyamides isolated from *S. oleoides* had weak cytotoxic effects against different cell lines although they showed good activities against fungi and bacteria (Arora *et al.,* 2014).

S. oleoides is not been fully studied for its bioactive compounds and mode of action needs to be addressed for its vast range of biological activities. Moreover, further pharmacological exploration is also needed.

Chapter 3

Materials and methods

3.1. Plant Material

S. Oleoides was collected from CIDS (Cholistan institute of desert studies), The Islamia University of Bahawalpur (IUB) and verified by Dr. Shazia Anjum (director CIDS).

S. persica was collected from a village "Goth Bahar" District Lodhran near Bahawalpur City and verified by Dr. Qasim Hayat (ASAB, NUST).

Roots and leaves of the Plants were dried under shade at room temperature. Dried parts of the plants were ground to fine powder in grinder.

3.2. Extraction

Ground fine powder of plant material was subjected to extraction using different solvents ensuring extraction at different polarities. Four different solvents were used namely ethanol, methanol, n-hexane and ethyl acetate. Weighed amount of plant material i.e. 10 gram was soaked in the solvent (100 ml) for 24 hours. Afterwards, solvent was filtered. Extracts solubilized in solvent were then evaporated to dryness in rotary evaporator under pressure. Temperature of the rotary evaporator was set below the boiling point (around 50°C of the respective solvent. The concentrated extracts were then dried in an incubator (Mermet GmbH, Germany) at 37°C for 72 hours as previously described by Yang *et al*., (2013). All of the extracts were stored in sealed petri dishes.

3.3. 2, 2-di-phenyl-2-picryl hydrazyl hydrate (DPPH) assay

The free radical scavenging ability of the plant extracts was evaluated using 2, 2-di-phenyl-2-picryl hydrazyl hydrate (DPPH). The method used was a modified method

previously described by Brand-Williams and co-workers in 1995. It has been observed that DPPH has an ability to interact with anti-oxidant compounds having the ability to donate hydrogen and then cause the reduction of DPPH. DPPH is a colorimetric substance, hence the visualized change in emission spectrum from deep violet to light yellow can be observed at 517nm using a Ultra Violet/ visible light spectrophotometer. A 10mM DPPH stock was prepared in ethanol. Working solution of 0.1mM concentration was prepared by doing further dilution. A single reaction consisted of 100 µl sample (dissolved in ethanol), 100 µl of DPPH solution (0.1mM) and 800 µl of solvent. Blank was prepared by adding 100µl DPPH and 900µl of solvent. The experiment was carried out in triplicate. The samples are incubated in darkness for 15-20 minutes at RTP, later the decreasing absorbance was evaluated. The ability of the compound to scavenge free radicals was evaluated using the formula:

% scavenging = [Absorbance of blank - Absorbance of test sample/Absorbance of blank] X 100.

$$\% \text{ Inhibition} = [(A_b - A_s)/A_b] \times 100$$

Values were plotted on a graph to compare relative scavenging abilities. A standard test was also performed with Ascorbic acid

3.4. Culturing HuH-7 cell line

Human Hepatocellular carcinoma cell line (Huh-7) was obtained from cell culture bank at ASAB NUST. The cell lines were grown and maintained in Dulbecco's Modified Eagle Media (High Glucose) (Sigma Aldrich, USA)**.**

3.4.1. Media preparation

DMEM powder (14.7 gm) (Sigma Aldrich, USA) was dissolved in 850ml double distilled autoclaved water. Sodium bicarbonate (3.7 gm.) was added to the solution until

the color changed to dark pink. The addition of Sodium bicarbonate ensured the attainment of optimum pH of 7.2-7.4 which was measured using the pH paper. Heat inactivated, sterile 10% Fetal Bovine Serum (FBS) (PAA laboratories) (100ml) was also added. Double distilled autoclaved water was used to make the final volume up to 1L. The media was filter sterilized and stored at 4ºC till further use. An antibiotic cocktail comprising penicillin and streptomycin (Sigma Aldrich, USA) was added (1% of the total media) to the prepared media.

A portion of the media was shifted to a 25cm^2 tissue culture flask and incubated at 37ºC overnight to check for contamination.

3.4.2. Sub-culturing of cell line

Growing cell culture was obtained in 25cm^2 tissue culture flask and observed for confluency under an inverted microscope. At about 80% confluency, the cells were proceeded for splitting. Media was discarded from the flask and 2ml autoclaved Phosphate Buffered Saline (PBS) (0.01M) was added. After PBS washing, 1ml Trypsin-EDTA (Gibco, Life technologies) was added to the flask. The flask was left at 37ºC for 5 minutes. Detachment of cells was ensured with mild tapping of the flask between palms of the hands to aid the release of cells into the media. Once considerable number of cells were suspended, 2ml fresh media was added. Media containing the cell suspension was transferred to 15ml falcon tube. The tube was centrifuged at 1,000 rpm for 5 minutes at 4ºC in a refrigerated centrifuge machine (5810R, Eppendorf, Germany). Supernatant was discarded and the pellets was re-suspended in 1ml DMEM containing 10% FBS. Cell suspension (0.5 ml) from the tube was shifted to two 25cm^2 tissue culture flasks. Prepared DMEM (5ml) was added to each flask and the flasks were placed at 37ºC in a 5% CO2 incubator.

The flasks were observed for confluency under an inverted microscope at 20X magnification power**.**

3.4.3. Cryopreservation

Cryopreservation of the cell line was done to provide a secure cell stock in case of an accidental loss and genetic or phenotypic instability. A cryoprotectant solution was formulated comprising 9 parts Fetal Bovine Serum (FBS) and 1 part Dimethyl sulfoxide (DMSO) (10%, Merck, Germany) and a total volume of 10ml mixture was prepared**.** The cell suspension left after splitting the cells was mixed with the cryoprotectant solution. Afterwards 1.5 of the mixture of cell suspension and cryoprotectant solution was dispensed in cryovials. The vials were placed on ice for 10-15 minutes and then at -20°C for 2 hours before being finally shifted to - 80°C for long term storage.

3.4.4. Revival of cryopreserved cell lines

Cryovial containing the cell suspension and cryoprotectant solution was revived firstly by placing it on ice to thaw the constituents and then the vial constituents were added to 5 ml media placed in 15ml falcon. The falcon was then proceeded for centrifugation at 4°C at 1,000 rpm for 5 minutes. The supernatant containing the cryoprotectant and media was discarded. The cells were resuspended in 1ml growth media (DMEM containing 10% FBS) and then seeded into a 25cm^2 tissue culture flask containing 5ml growth media. The flasks were checked periodically to record the growth rates and confluency percentage under an inverted microscope at various magnifications.

3.5. Sample preparation for MTT and antibacterial assay

A stock solution of each extract was prepared in DMSO keeping the concentration 50mg/ml. Stock solution was then filter sterilized using 0.2μm syringe filter.

3.6. MTT Assay

5mg/ml MTT solution was made in phosphate buffer saline (PBS). The solution was filtered through a 0.2 um filtered and stored at 0-4°C.

100ul of cells (10,000 cells in number) were added into each well of 96 well plates and incubated for 24 hours. Cells were then incubated with varying concentrations of extracts for 24 hours and 72 hours at 37°C. Then 20µl of MTT Reagent was added to each well, including controls. The plate was incubated in a cell culture incubator for 3 hours until a purple precipitate was visible. Then 100µl of DMSO was added to all wells, including controls. The plate was left covered at room temperature in the dark for 1 hour and then absorbance of each well, including the blanks were recorded at 570 nm in a microplate reader. The average values from triplicate readings were determined and the average value of the blank was subtracted from it. Cell viability was calculated and converted to percentage viability. Percentage viability was then plotted against increasing concentrations of extracts.

3.7. Antibacterial activity

3.7.1. Collection of Bacterial Strains

Three clinical drug resistant bacterial strains of *Salmonella typhi, Staphylococcus aureus* and *Pseudomonas aeruginosa* were provided by Phage research group of Department of Industrial Biotechnology, ASAB. These bacterial strains were isolated from samples of patients in Armed Forces Institute of Pathology (AFIP), Rawalpindi. At the time of collection of samples from patients, their name, age, gender and source was recorded along with any other medical complications present. Isolation of bacterial strains was carried from specimens of pus/swabs. These specimens were then treated according to the standard protocol that is followed by AFIP. Bacterial strains were isolated by growing them in differential media. Single colonies of bacteria were picked

from agar and further treated for evaluation of their antibiotic resistance profile. Pure bacterial strains were then collected in 50% glycerol stock solutions and stored at 80 °C.

3.7.2. Preparation of Media

Nutrient Agar (Merk) was prepared in distilled water (28 g/L) and sterilized autoclaving according to manufacturer's instructions prior to use. Autoclaved petri plates were poured with liquid agar inside laminar flow hood cabinet (StreamLine laboratory product, Singapore) for avoiding any contamination. Agar plates were then covered, sealed and incubated overnight at 37 °C for checking the sterility of plates before further experimentation.

3.7.3. Inoculation of Bacterial Strains

Bacterial strains stored in glycerol stock solutions were streaked on Nutrient agar media and allowed to grow in colonies. Standard inoculation procedures were carried out using inoculation loops and petri plates containing bacterial samples which were then kept in incubator for 16 hours at 37 °C.

3.7.4. Antibacterial Disc Diffusion Assay

Bactericidal ability of extracts was estimated through disc diffusion assay. This assay was performed in laminar flow hood with UV turned on 15 minutes prior to each experiment to avoid any unwanted contamination. 5 mL solution of 0.9% normal saline was prepared and poured in sterile test tubes inside laminar flow hood. Single pure colonies of bacterial strains were picked from streaked plates using sterile loop and dipped in normal saline solution to prepare bacterial inoculum for the assay. Normal saline containing bacterial inoculum was then swabbed on nutrient agar plates using sterile cotton swabs. To ensure even spreading of inoculum on agar media, plates were rotated 60 degree three times. Filter paper discs of 6 mm diameter were prepared from

Whattman Filter Paper Grade 4 and contained in a petri plate, followed by sterilization by autoclave. Sterilized filter paper discs were then placed on inoculated plates by using a sterile syringe (BD 5 mL syringe, Becton and Dickinson, Pakistan) at points that were already marked for each dilution of extract. 10 µl of each dilution of extract was then dispensed on swabbed plates and allowed for absorption by filter paper discs. Deionized water was used as a negative control for disc diffusion assay. After setting up plant extract impregnated discs for disc diffusion assay, these plates were incubated overnight at 37 °C and each experiment was repeated twice to ensure validation of the assay.

3.8. Molecular Docking studies

The selected phytochemicals under study, were docked against HCV proteins to screen phytochemicals of *Salvadora prersica* and *Salvadora oleoides* to find a potent drug candidate that can inhibit viral infection effectively. These compounds were also docked against proteins primarily involved in developing resistance against various classes of antibiotics. For the purpose, Autodock Vina (version 1.1.2) was used.

3.8.1. Protein preparation

Autodock Vina helps in determining the binding affinity of ligand molecule with protein. By default, 9 different confirmations are generated for each analysis. Tertiary structures of Proteins were retrieved from RCSB protein databank and PDBIDs were saved. Discovery studio (4.1.0) was used to visualize and modify the proteins. Proteins are saved in pdb format. AutoDock Tools (1.5.6) was then used to prepare proteins for docking by protonation and energy minimization of receptor proteins. We can select the search space by setting parameters in grid box. After setting all parameters proteins were saved in pdbqt format and analyzed by autodock vina for ligand binding conformations.

3.8.2. Ligand preparation

ChemSketch (11.02) was used to sketch ligand molecules and subjected to 3D optimization. Mol format generated by ChemSketch was converted to pdb format by using ArgusLab (4.0.1). After optimization of ligand in AutoDock tools, ligand structures were saved in pdbqt format.

3.8.3. Docking analysis

After ligand and receptor preparation, docking analysis was done using AutoDock Vina. Docking output was obtained in the form of binding energy (kcal/mol) and a binding pose file. Lower value showed the more stable binding conformation. On the basis of binding energy best pose file for each ligand-protein binding was selected. Discovery studio was utilized for visualization of best pose and 2D and 3D poses were generated.

3.9. Statistical Analysis

During the compilation of data Microsoft Office Excell Worksheet was used. For statistical evaluation the Statistical Package GraphPad PRISM Ver. 5.01 Software was used. One way ANOVA was the statistical analysis of choice with Post-HOC analysis using Bonferroni posttest where comparisons with control and among groups was required. Descriptive statistics using cross-tabulation were used to evaluate Data and T-tests were used for group-group significance and testing of hypotheses. A 95% Confidence interval was used throughout, and all values having a p value showing $p<0.05$ were taken to be statistically significant.

Chapter 4

Results

4.1. 2,2,-Di-Phenyl-2-Picryl Hydrazyl Hydrate (DPPH) Assay

4.1.1. *S. oleoides* leaves

For all concentrations the percentage inhibition of DPPH by ascorbic acid (positive control) was observed to be higher ($p<0.05$). Among the extracts, methanolic extract showed the highest free radical scavenging ability and it reached up to 65% at the concentration of 200 µg. Ethanolic extract showed the DPPH inhibition up to 55%. It can be seen that there is non-significant difference between the activities of methanolic and ethanolic extracts ($p>0.05$). There was no statistical difference between the activities of ethyl acetate and n-hexane as well ($p>0.05$).

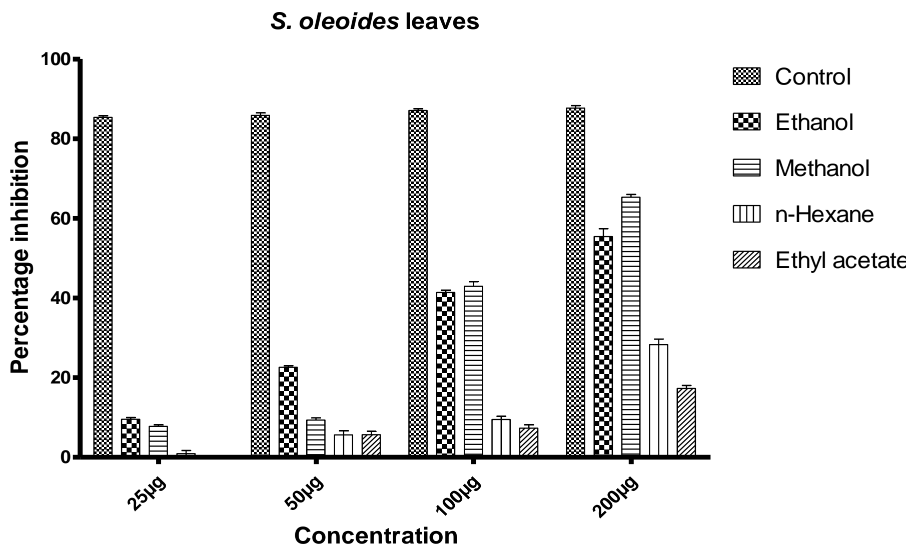


Figure 4.1 Showing DPPH Inhibition of Ascorbic Acid control and extracts at different concentrations of *S. oleoides* leaves ($p<0.05$)

4.1.2. *S. oleoides* roots

There was increase in inhibition of DPPH at increasing concentrations ($p<0.05$) with Ascorbic acid control showing the highest DPPH inhibition ($p<0.05$). Among the

extracts, a non-significant difference was found between the activities of ethyl acetate, ethanol and methanol ($p>0.05$) with highest activities of 61%, 54% and 45% respectively at the concentration of 200 µg. n-Hexane showed maximum inhibition up to 20%.

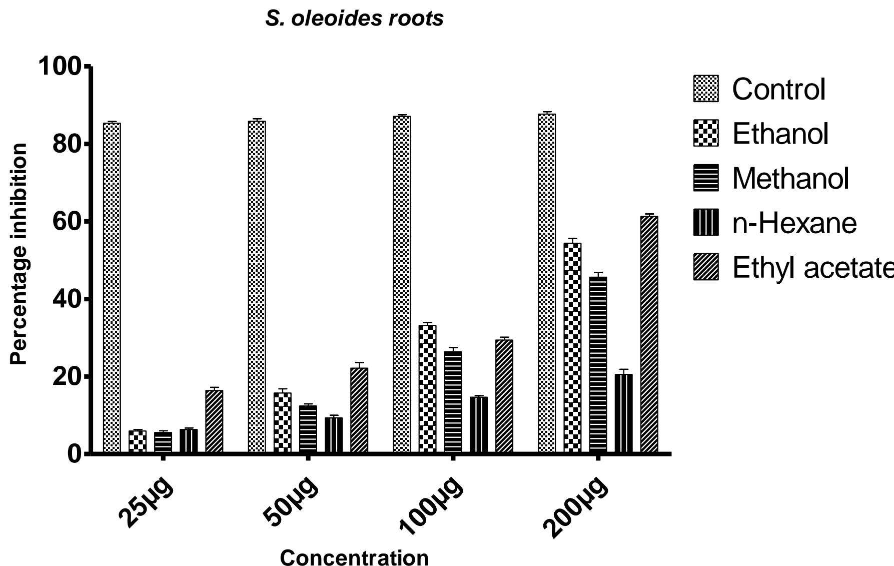


Figure 4.2 Showing DPPH Inhibition of Ascorbic Acid control and different extracts of *S. oleoides* roots ($p<0.05$)

4.1.3. *S. persica* leaves

Ethanolic extract of *S. persica* leaves inhibited DPPH up to 38% and methanolic extract inhibited DPPH up to 30%. Statistical analysis revealed non-significant difference among the different extracts of *S. persica* leaves ($p>0.005$). A significant difference of $p<0.001$ was found between the activity of Ascorbic Acid control and different extracts. Ascorbic acid showed the activity up to 87%.

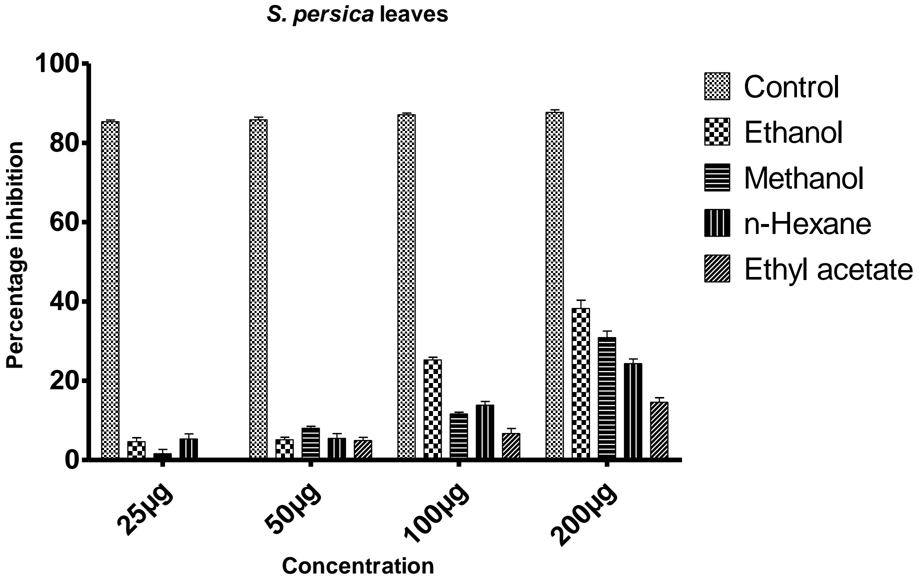


Figure 4.3 Showing DPPH Inhibition of Ascorbic Acid control and different extracts of *S. persica* leaves (p<0.05)

4.1.4. *S. persica* roots

A non-significant difference (p>0.05) was seen among the free radical scavenging activities of ethanol, methanol and ethyl acetate extracts. 76 %, 64% and 63% were the maximum activities shown by ethyl acetate, ethanolic and methanolic extracts respectively. n-Hexane extract had no activity at 25 µg and at a concentration of 200 µg it inhibited 20% of DPPH.

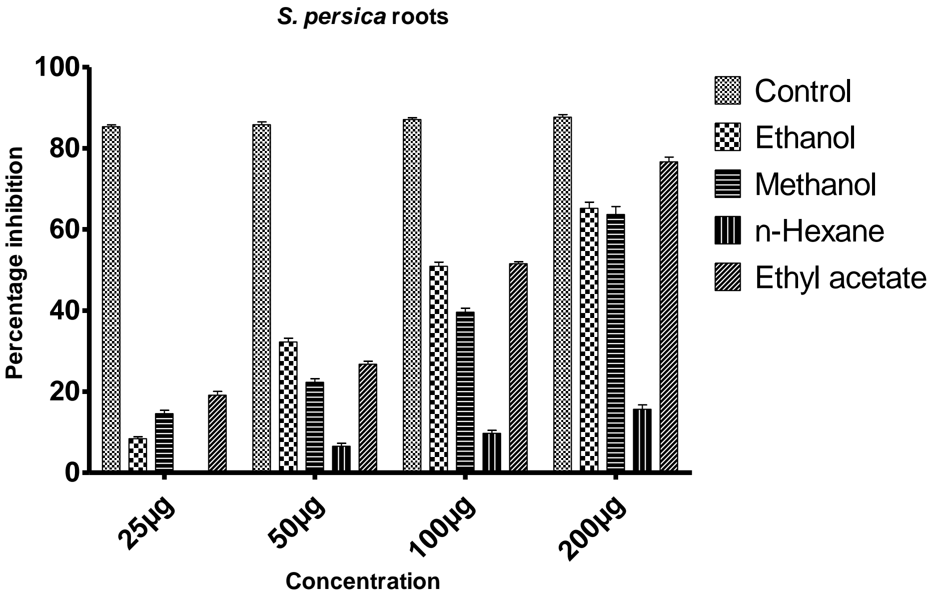


Figure 4.4 Showing DPPH Inhibition of Ascorbic Acid control and different extracts of *S. persica* roots ($p<0.05$)

4.2. MTT (methyl thiazol tetrazolium) Assay

Toxicological effects of different extracts of leaves and roots of both plants were determined through MTT proliferation assay. Succinic dehydrogenase in living cells reduces MTT to purple formazan crystals. Formazan crystals are solubilized in DMSO and absorption in the visible range correlates with cell viability. Figures 4.5, 4.6, 4.7 and 4.8 demonstrate that cell proliferation of HuH-7 cell line is unaffected up to higher concentrations.

In case of *S. oleoides* no extract showed 50% reduction in the cell viability except ethanolic leaf extract, which reduced the cell viability up to 48%, 47% and 44% at the concentration of 250 µg, 500 µg and 1000 µg respectively.

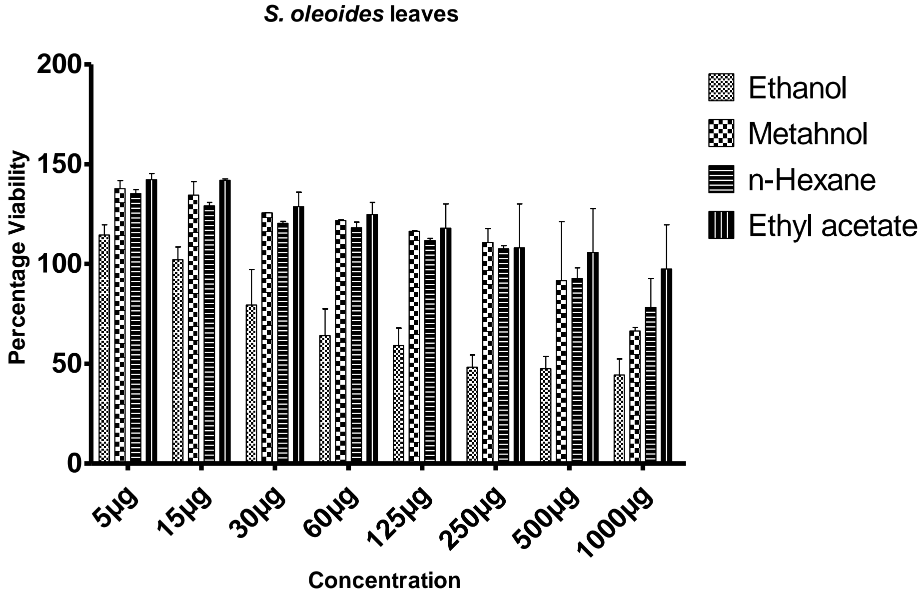


Figure 4.5 Showing the percentage inhibitory effect of different extracts of *S. oleoides* leaves in a dose dependent manner on HuH -7 cell line ($p<0.05$). There is non-significant difference in the inhibitory effect of methanol, n-Hexane and ethyl acetate extracts ($p>0.05$).

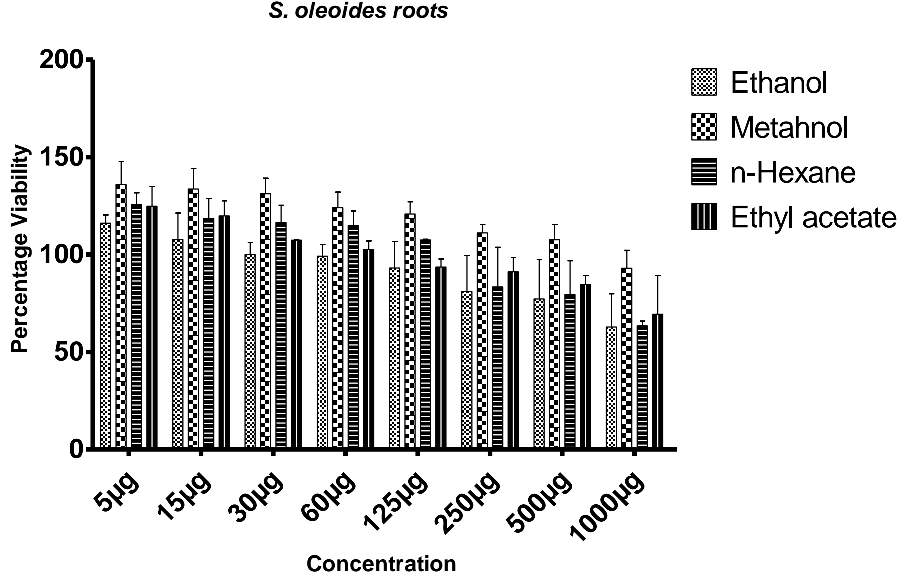


Figure 4.6 Showing the percentage inhibitory effect of different extracts of *S. oleoides* roots in a dose dependent manner on HuH -7 cell line ($p<0.05$). A significant difference was seen in the activity of ethanolic and methanolic extracts ($p<0.05$), where a non-significant difference was observed between other extracts ($p>0.05$).

In case of *S. persica*, no root extract showed 50% reduction in cell viability however, different leaf extracts reduced the cell viability below 50% at higher concentrations. Ethanolic extract reduced the cell viability up to 43% at the concentration of 1000 µg. Methanolic extract of the *S. persica* leaves was most lethal. It reduced the cell viability up to 28%, 39%, 44% and 46% at 1000 µg, 500 µg, 250 µg and 125 µg concentration respectively.

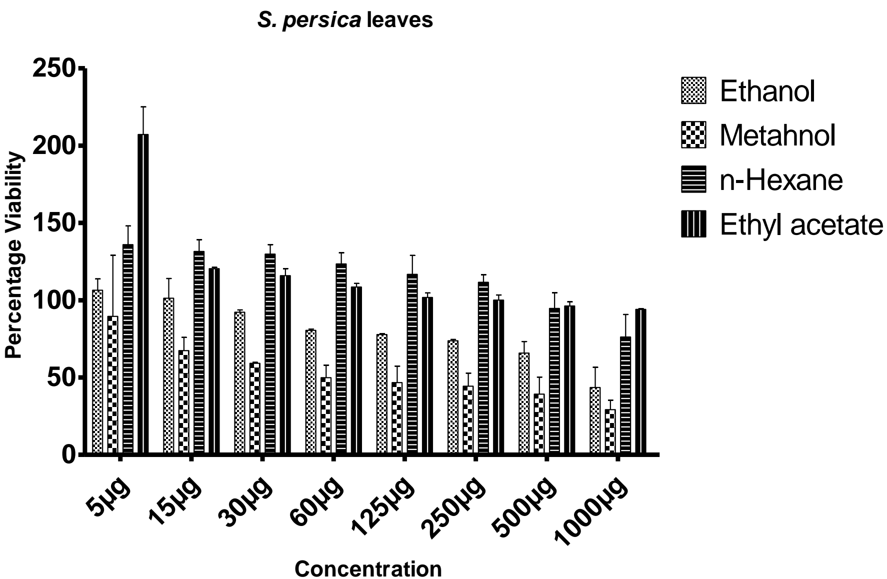


Figure 4.7 Showing the percentage inhibitory effect of different extracts of *S. persica* leaves in a dose dependent manner on HuH -7 cell line ($p<0.05$). $p>0.05$ (non-significant) for ethanol vs methanol, ethanol vs n-hexane and n-hexane vs ethyl acetate.

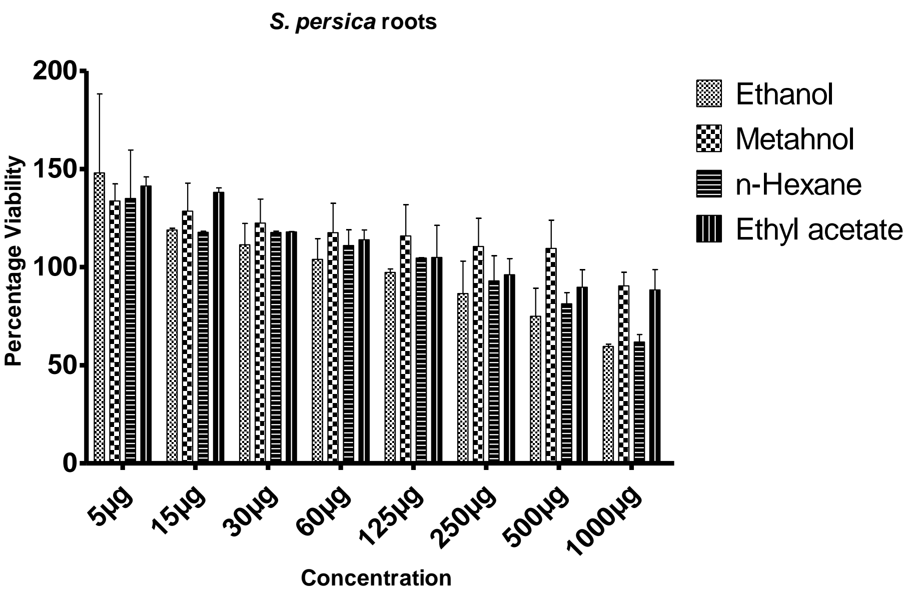


Figure 4.8 Showing the percentage inhibitory effect of different extracts of *S. persica* leaves in a dose dependent manner on HuH -7 cell line ($p>0.05$). No significant difference was observed among the activity of different extracts.

4.3. Antibacterial activity

Antibacterial activity of extracts was measured by disc diffusion method. Observations were recorded in the form of millimeter (mm) clearing zone. All the values of clearing zone are given in appendix. Figures 4.9-4.20 are demonstrating the antibacterial activity of different extracts of *S.persica* and *S. oleoides* plants against three multi-drug resistant strains of bacteria naming *Salmonella typhi*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. These strains were already evaluated for their antibiotic susceptibility and were found to be totally resistant against erythromycin and penicillin. All the extracts showed moderate anti-bacterial activity against these drug resistant strains in dose dependent manner by showing zones of inhibition of increasing diameter with increasing concentration of extracts.

Maximum diameter of zone of inhibition was found to be approximately 16 mm, signifying the efficacy of extracts against antibiotic resistant strains of bacteria. Minimum diameter of zone of inhibition was observed to be 6mm. There was no zone of inhibition around filter paper discs that were treated with de-ionized water, acting as negative control. So, it can be concluded that the zones of inhibition observed in discs treated with extracts was actually due to presence of extracts.

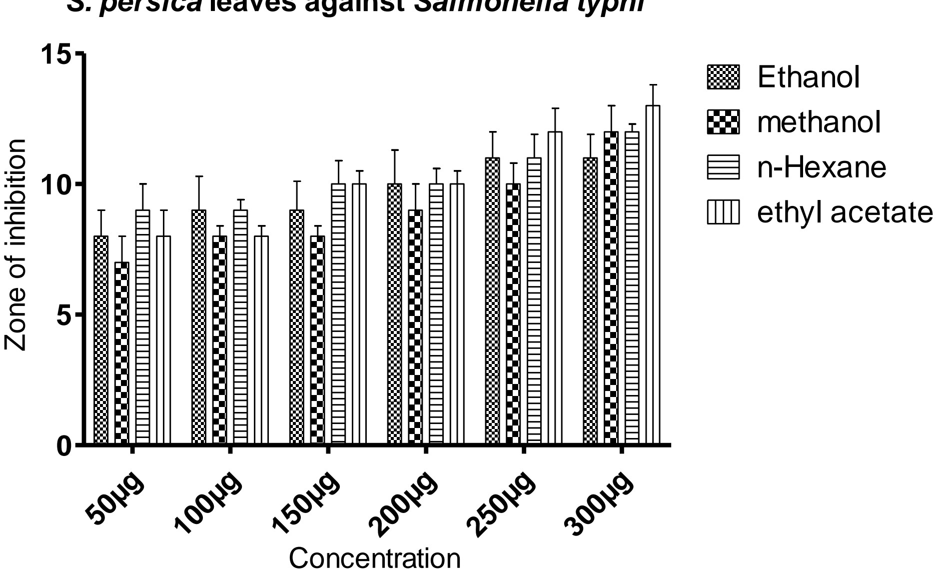


Figure 4.9 Difference in clearance zone values (mm) of all extracts of *S. persica* leaves against *Salmonella typhi* at different concentrations (p>0.05). Range=7-13 mm

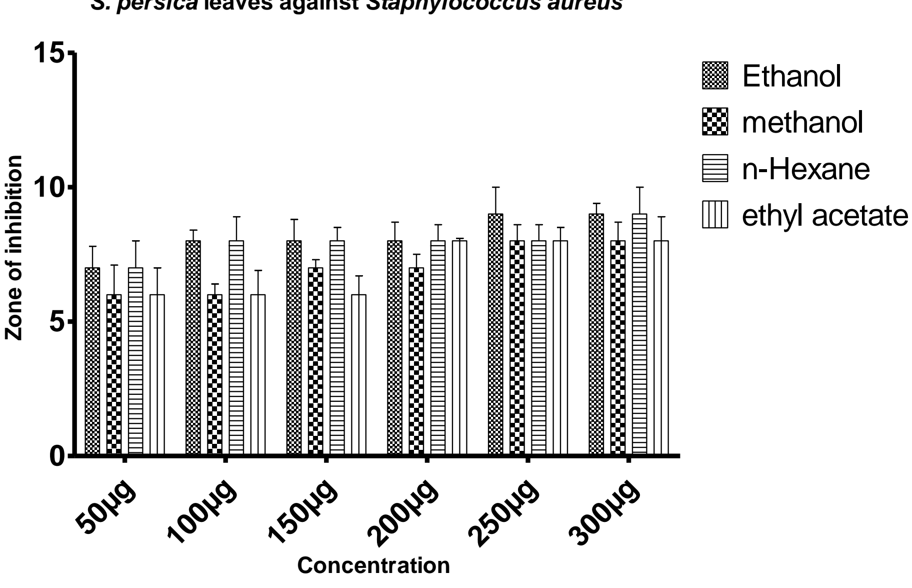


Figure 4.10 Difference in clearance zone values (mm) of all extracts of *S. persica* leaves against *Staphylococcus aureus* at different concentrations (p>0.05). Range=6-9 mm

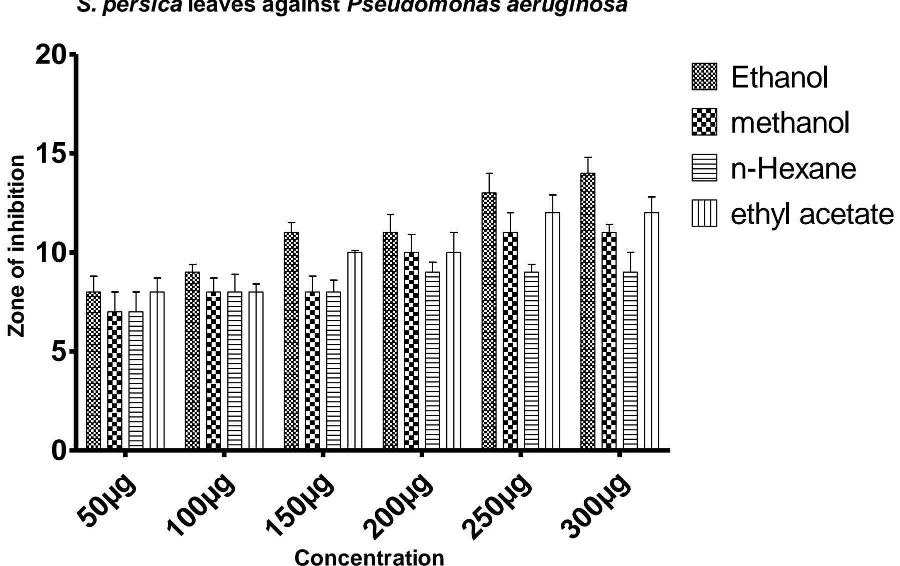
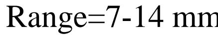


Figure 4.11 Difference in clearance zone values (mm) of all extracts of *S. persica* leaves against *Pseudomonas aeruginosa* at different concentrations (p>0.05).


Range=7-14 mm

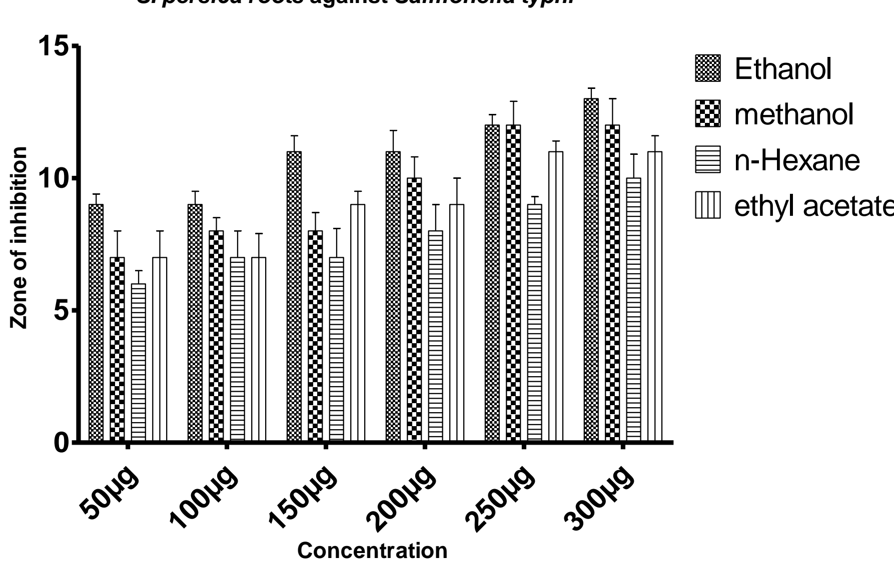


Figure 4.12 Difference in clearance zone values (mm) of all extracts of *S. persica* roots against *Salmonella typhi* at different concentrations (p>0.05). Range=7-13 mm

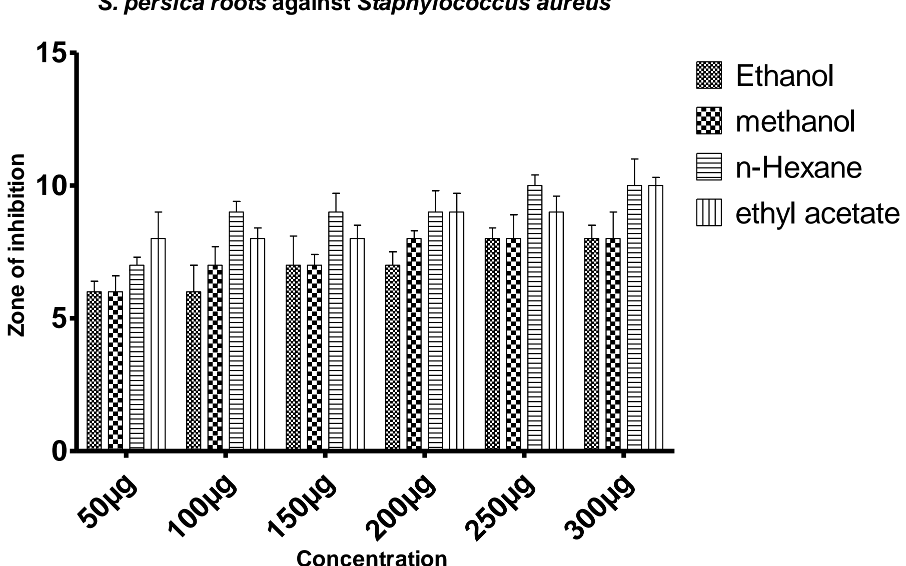


Figure 4.13 Difference in clearance zone values (mm) of all extracts of *S. persica* roots against *Stphylococcus aureus* at different concentrations (p>0.05). Range=6-10 mm

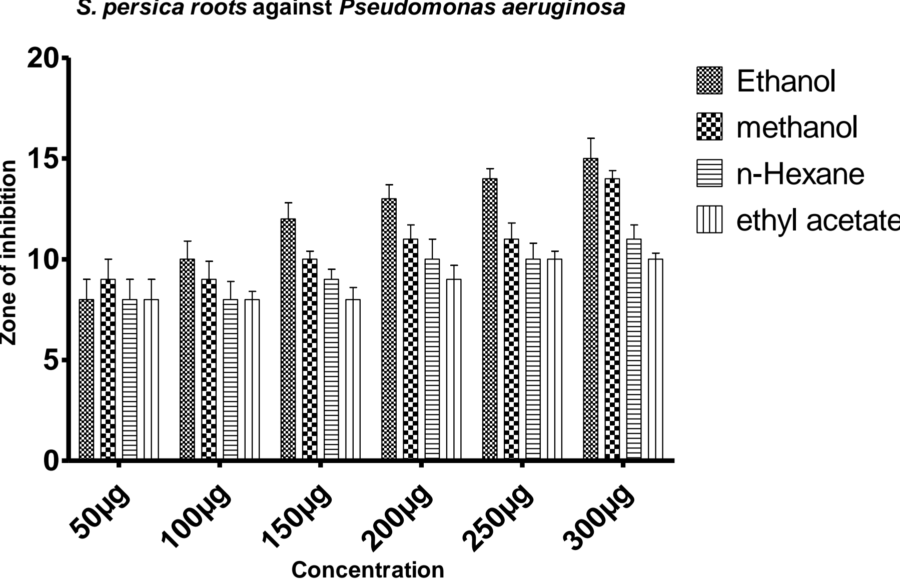


Figure 4.14 Difference in clearance zone values (mm) of all extracts of *S. persica* roots against *Pseudomonas aeruginosa* at different concentrations (p>0.05). Range= 8-15 mm

S. oleoides **leaves against** ***Salmonella typhi***

Zone of inhibition

15
10
5
0

Ethanol
methanol
n-Hexane
ethyl acetate

50µg
100µg
150µg
200µg
250µg
300µg

Concentration

Figure 4.15 Difference in clearance zone values (mm) of all extracts of *S. oleoides* leaves against *Salmonella typhi* at different concentrations (p>0.05). Range= 8-13 mm

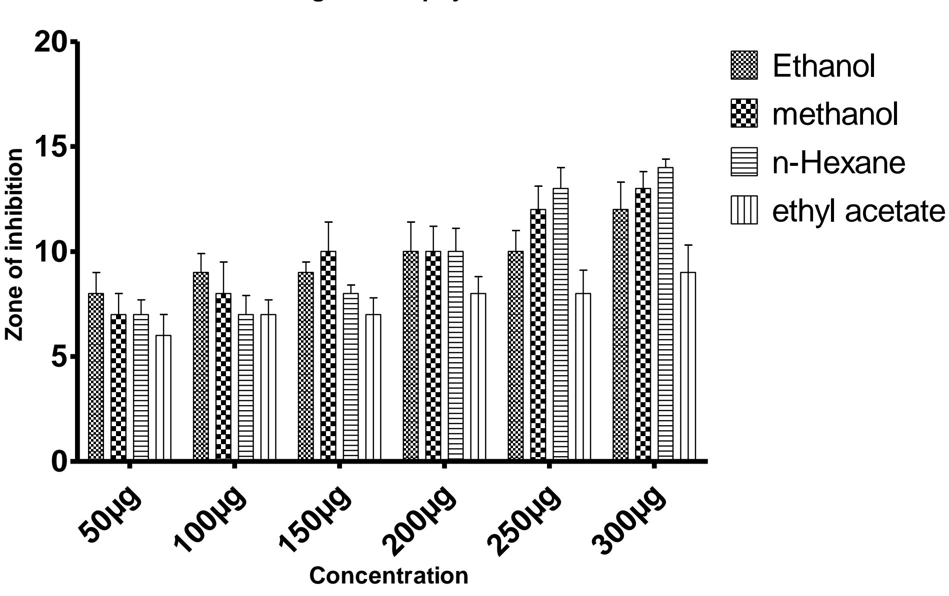


Figure 4.16 Difference in clearance zone values (mm) of all extracts of *S. oleoides* leaves against *Staphylococcus aureus* at different concentrations (p>0.05). Range= 6-14 mm

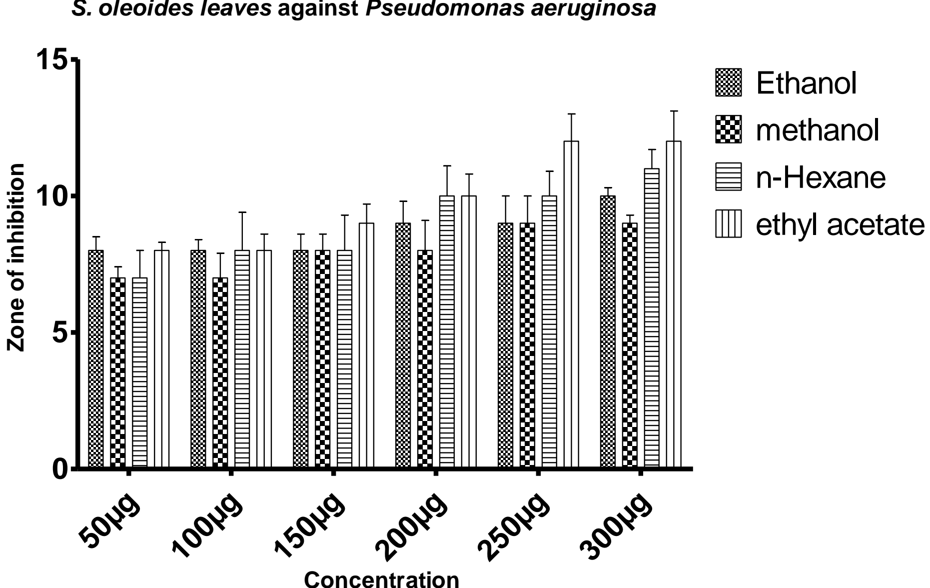


Figure 4.17 Difference in clearance zone values (mm) of all extracts of *S. oleoides* leaves against *Pseudomonas aeruginosa* at different concentrations (p>0.05). Range= 7-12 mm

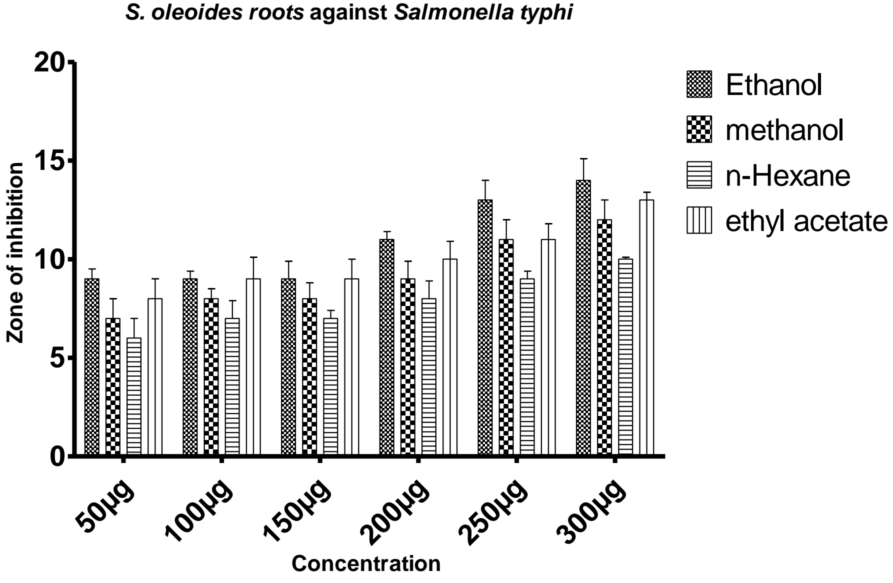


Figure 4.18 Difference in clearance zone values (mm) of all extracts of *S. oleoides* roots against *Salmonella typhi* at different concentrations (p>0.05). Range= 6-14 mm

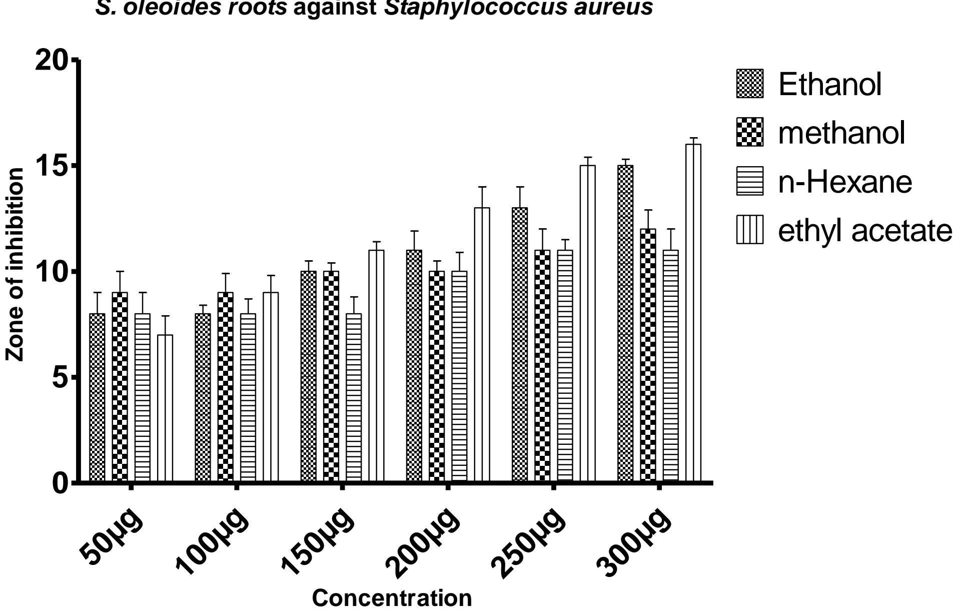


Figure 4.19 Difference in clearance zone values (mm) of all extracts of *S. oleoides* roots against *Staphylococcus aureus* at different concentrations (p>0.05). Range= 7-16 mm

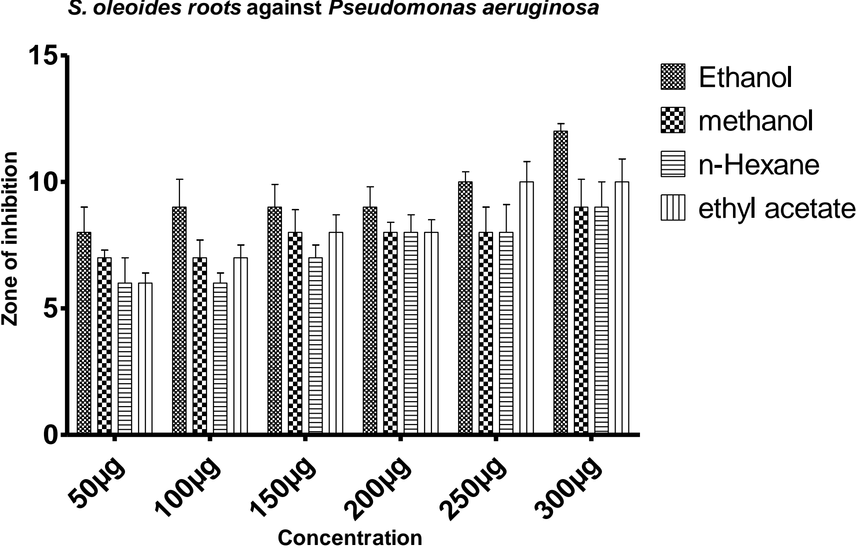
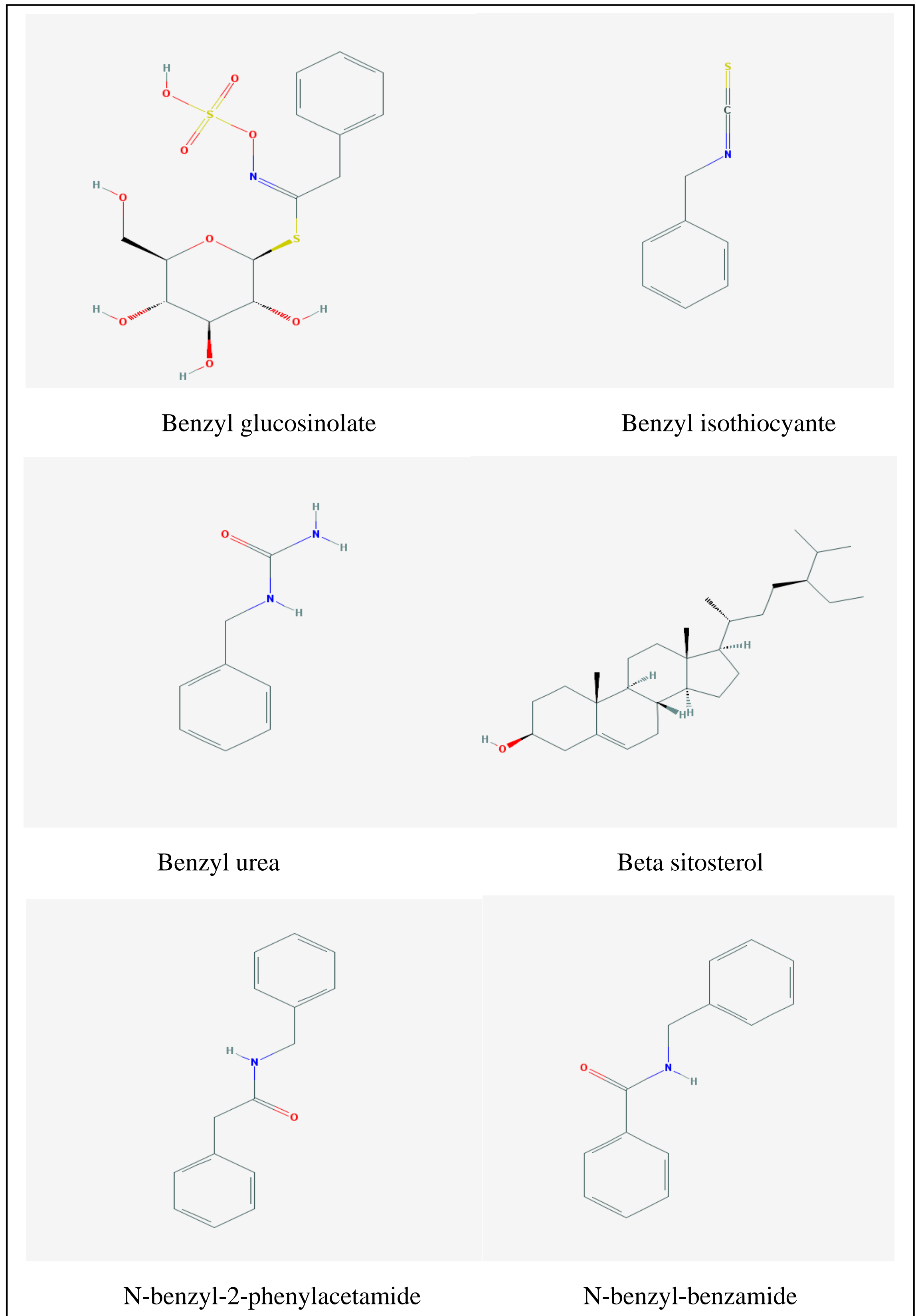


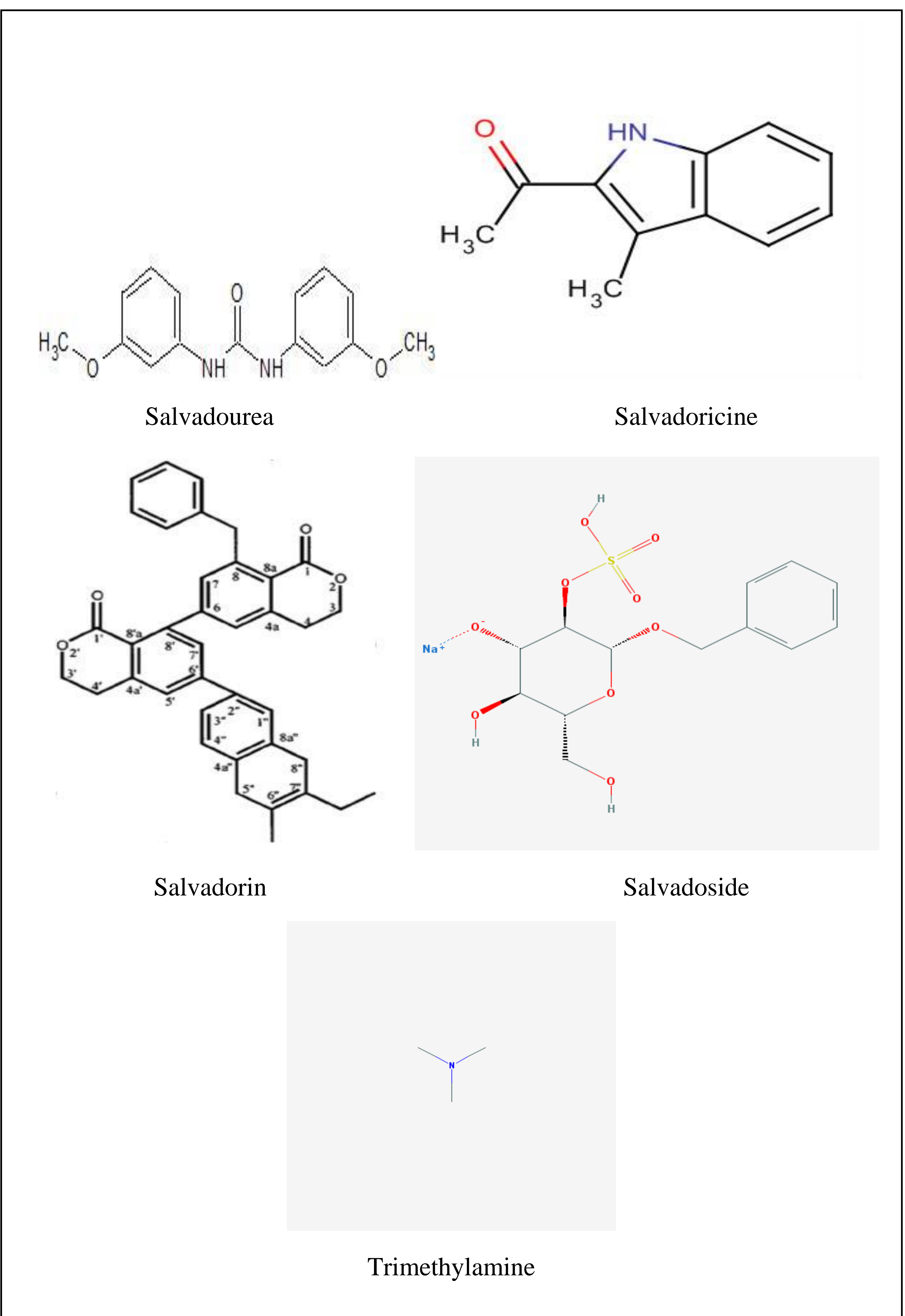
Figure 4.20 Difference in clearance zone values (mm) of all extracts of *S. oleoides* roots against *Pseudomonas aeruginosa* at different concentrations (p>0.05). Range= 6-12 mm

4.4. Molecular docking

Some bacterial multidrug resistance (MDR) causing proteins and 2 HCV proteins were selected for molecular docking study. Binding energies of selected compounds were calculated in AutoDock Vina. Binding energy values are given in table. Interaction was observed by using discovery studio (v .3.5) visualizer and Chimera (version 1.9).

Table 4.1 Showing structures of selected phytochemicals





Salvadourea

Salvadoricine

Salvadorin

Salvadoside

Trimethylamine

4.4.1. Docking against Bacterial multi drug ressstance causing proteins

Staphylococcus aureus, Salmonella typhi and *Pseudomonas aeruginosa* have many proteins that contribute to drug resistance. MexB, Sav 1866 and Amp-C were selected for docking analysis with the selected compounds. There binding affinities in terms of binding energies are summarised in the table:

Table 4.2 Showing binding energies of phytochemicals with drug resistance causing proteins

Ligand	Receptor (binding energy kcal/mol)		
	MexB	**Sav1866**	**Amp-C**
Benzyl glucosinolate	-7.8	-8.1	-6.8
Benzyl isothiocyanate	-5	-4.9	-4.9
Benzylurea	-6.2	-5.8	-5.5
N-Benzyl-2-phenylacetamide	-8.3	-8	-7.7
N-benzyl-benzamide	-7.9	-7.8	-7.3
Salvadoside	-6.4	-7.4	-6.5
Beta sitosterol	-6.7	-7.7	-6.6
Trimethylamine	-2.2	-2.4	-2.4
Salvadoricine	-7	-5.9	-7
Salvadorin	-14.5	-14.9	-10.5
salvado urea	-7.1	-7.7	-7.6
Penicillin	-7.4	-8.2	-8

Salvadorin is the compound with the most stable binding with all target proteins. Other compounds also resulted in good binding scores.

4.4.1.1. MexB

Pseudomonas aeruginosa muti-drug exporter MexB (PDBID 2V50) is the member of exporter proteins which are invoved in multi drug resistance in many organisms.

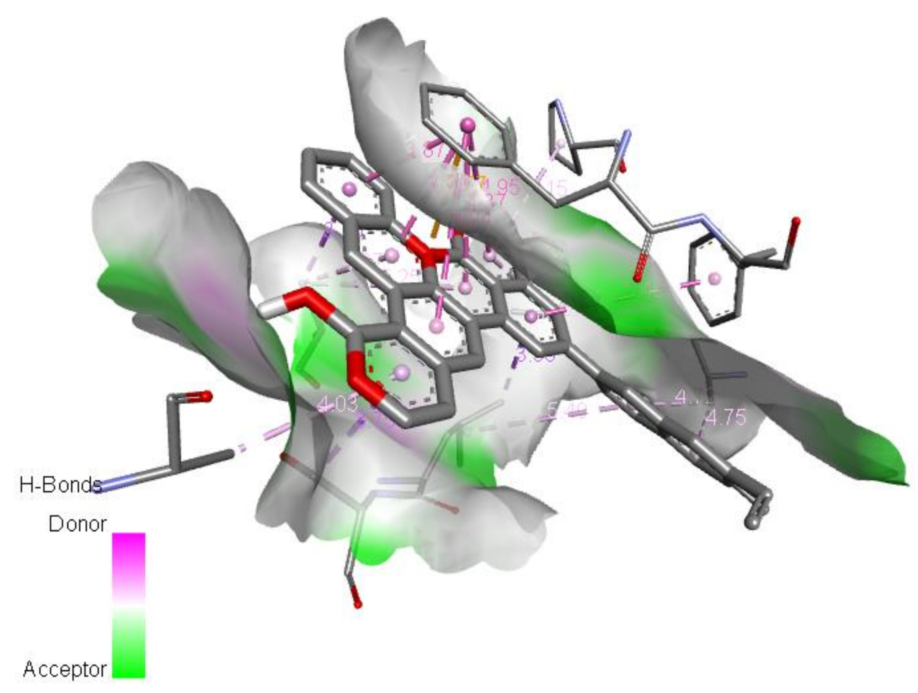


Figure 4.21 Showing Interaction of Salvadorin in binding pocket of MexB (3D image)

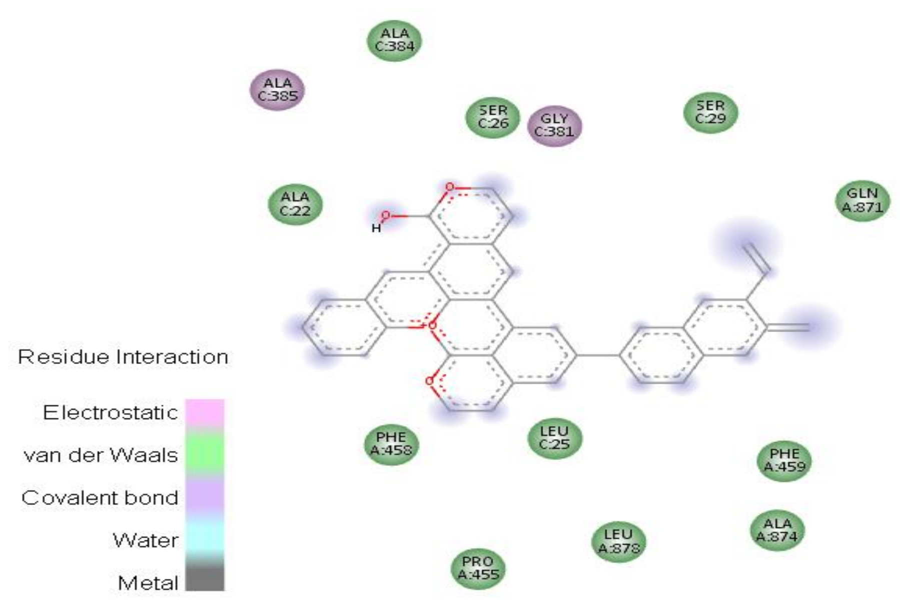


Figure 4.22 Showing Interaction of Salvadorin in binding pocket of MexB (2D image)

4.4.1.2 Sav1866

Staphylococcus aureus Sav1866 (PDBID 2ONJ) is an ABC transporter and causes multidrug resistance.

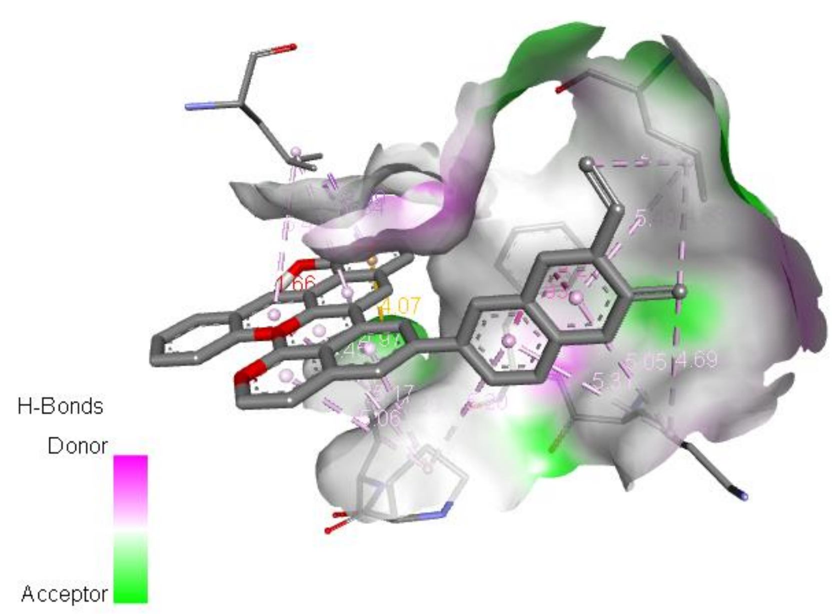


Figure 4.23 Showing Interaction of Salvadorin in binding pocket of Sav1866 (3D image)

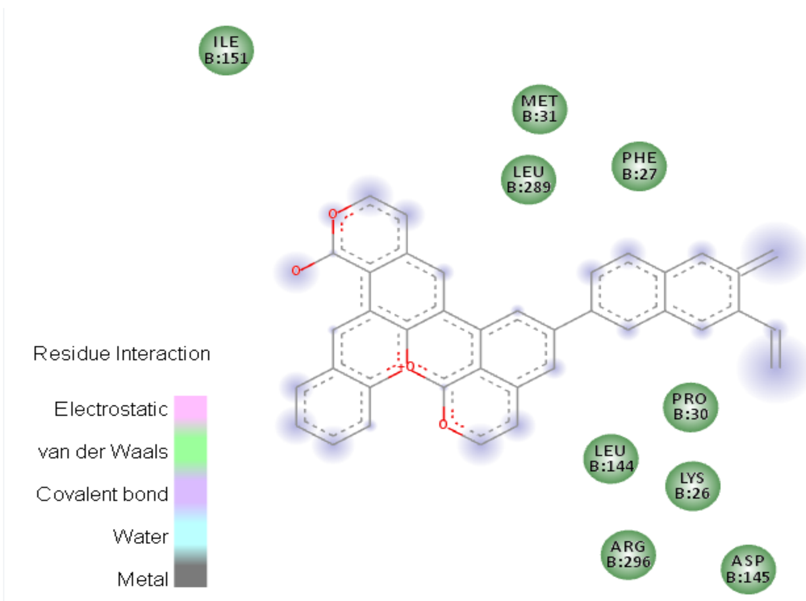


Figure 4.24 Showing Interaction of Salvadorin in binding pocket of Sav1866 (2D image)

4.4.1.3 Amp-C

Amp-C protein is involved in causing resistance against cephazolin and cefoxitin. Amp-C (PDB ID 1FSW) was selected and molecular docking study.

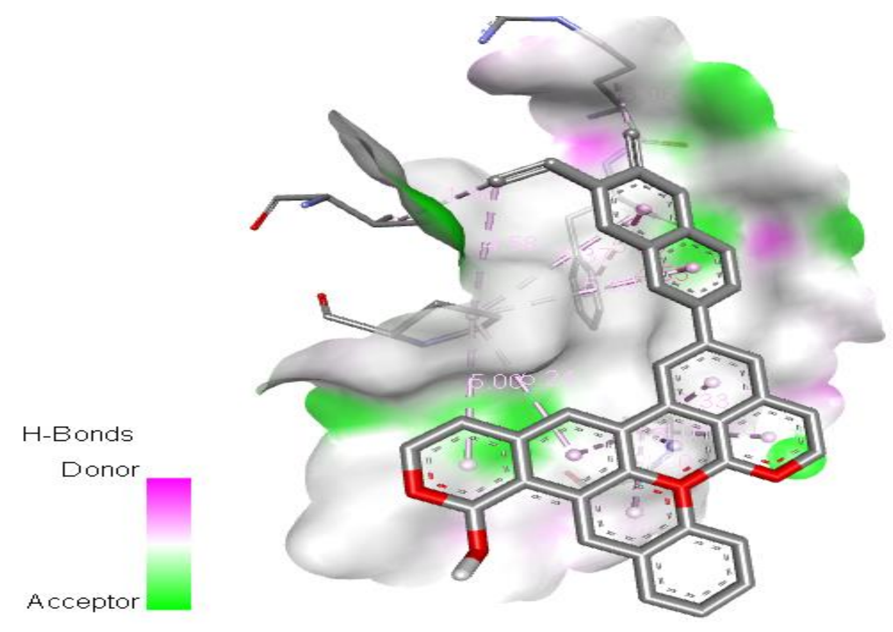


Figure 4.25 Showing Interaction of Salvadorin in binding pocket of Amp-C (3D image)

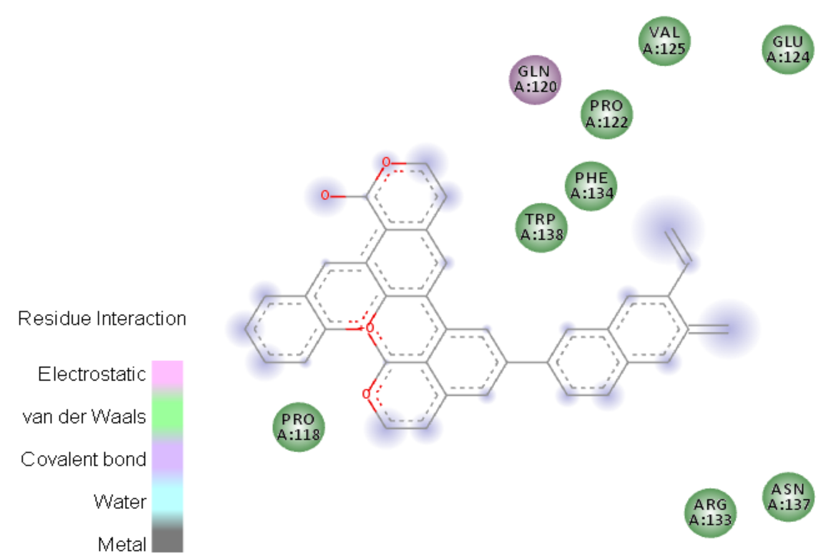


Figure 4.26 Showing Interaction of Salvadorin in binding pocket of Amp-C (2D image)

4.4.2. Docking against HCV proteins

2 HCV proteins NS3-4A (PDBID 3LOX) Protease and NS5B polymerase ((PDBID 3PHE) were selected for molecular docking study.

Table 4.3 Showing binding energies of phytochemicals with HCV NS3/4A protease and NS5B polymerase

ligands	**Receptors (Bindinding energy kcal/mol)**	
	NS3-4A Protease domain	**NS5B polymerase**
Benzyl glucosinolate	-6.5	-7.6
Benzyl isothiocyanate	-4.4	-5
Benzylurea	-5.6	-5.7
N-Benzyl-2-phenylacetamide	-5.88	-7.9
N-benzyl-benzamide	-6.2	-7.2
Salvadoside	-5.6	-6.1
Beta sitosterol	-6	-8.8
Trimethylamine	-2.3	-7.1
Salvadoricine	-6.3	-5.8
Salvadorin	-9.9	-12
salvado urea	-6.2	-2.2
Telaprevir*	-6.4	
Sofosbuvir**		-7.8

* Control drug for NS3/4A protease

** Control drug for NS5B polymerase

It can be seen that most compounds have good binding energy values compared to standard. Salvadorin has the lowest binding energy i.e. it shows the most stable binding with HCV protease.

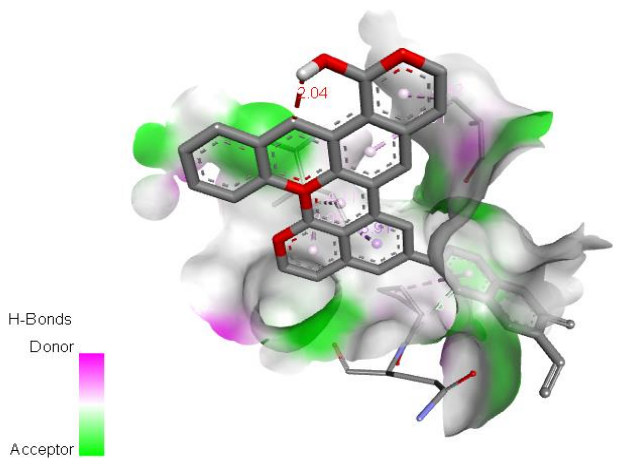


Figure 4.27 Showing Interaction of Salvadorin in binding pocket of NS3/4A protease (3D image)

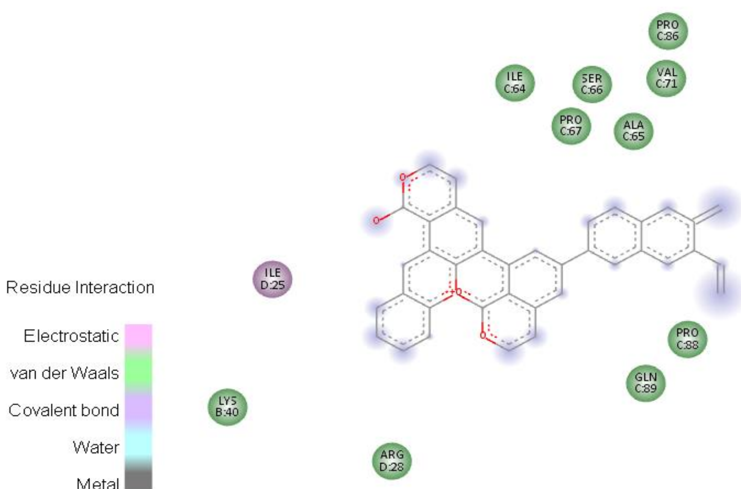


Figure 4.28 Showing Interaction of Salvadorin in binding pocket of NS3/4A protease (2D image)

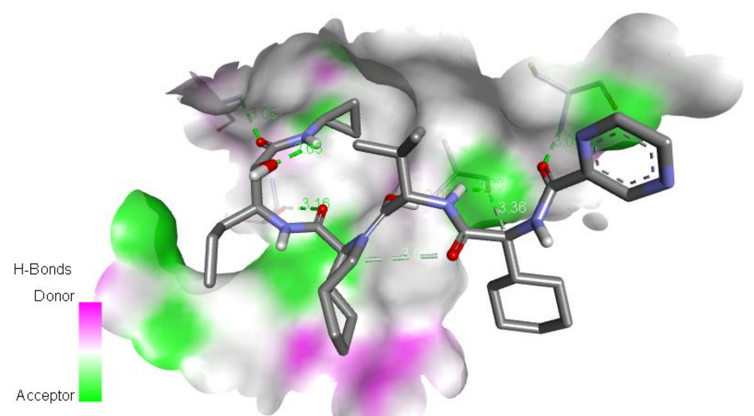


Figure 4.29 Showing Interaction of Telaprevir in binding pocket of NS3/4A protease (3D image)

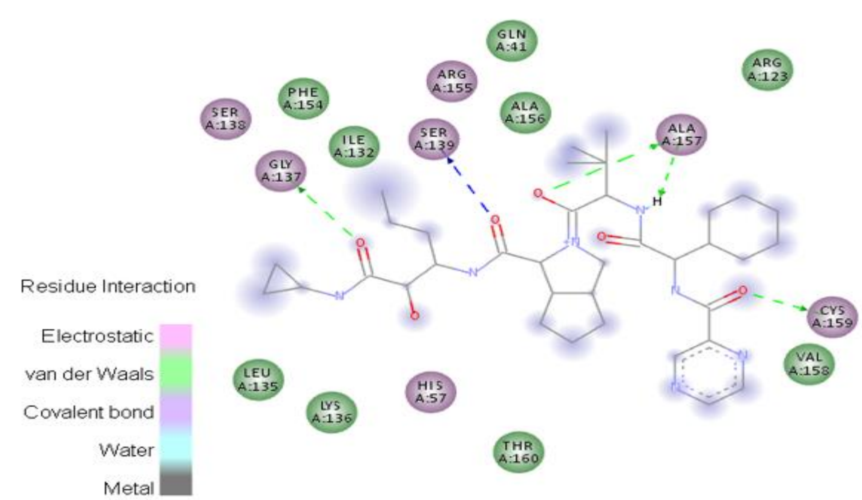


Figure 4.30 Showing Interaction of Telaprevir in binding pocket of NS3/4A protease (2D image)

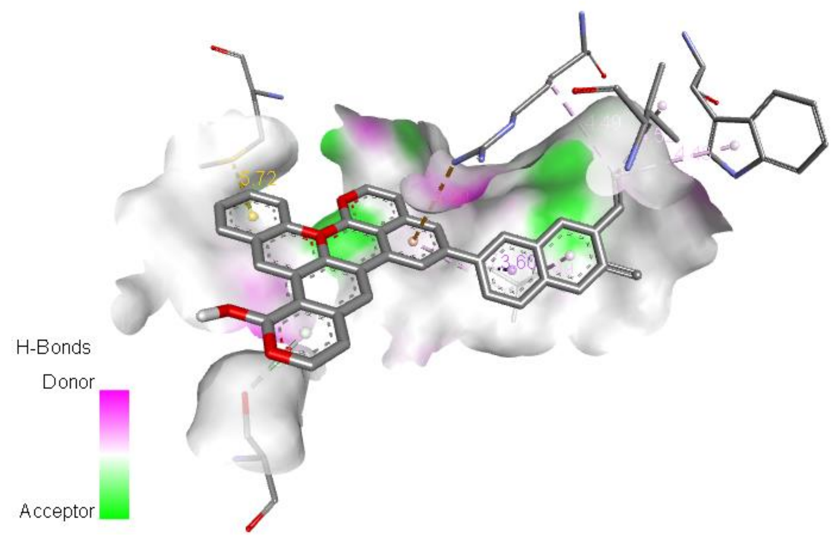


Figure 4.31 Showing Interaction of Salvadorin in binding pocket of NS5B polymerase (3D image)

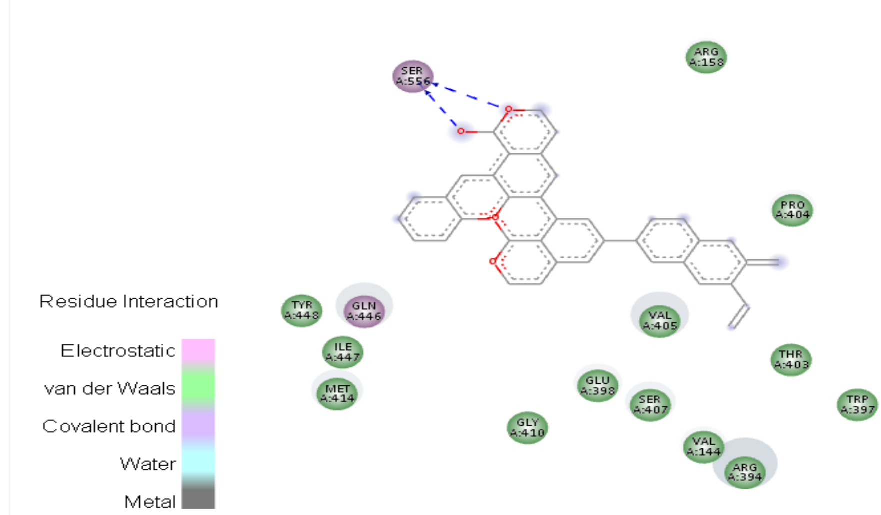


Figure 4.32 Showing Interaction of Salvadorin in binding pocket of NS5B polymerase (2D image)

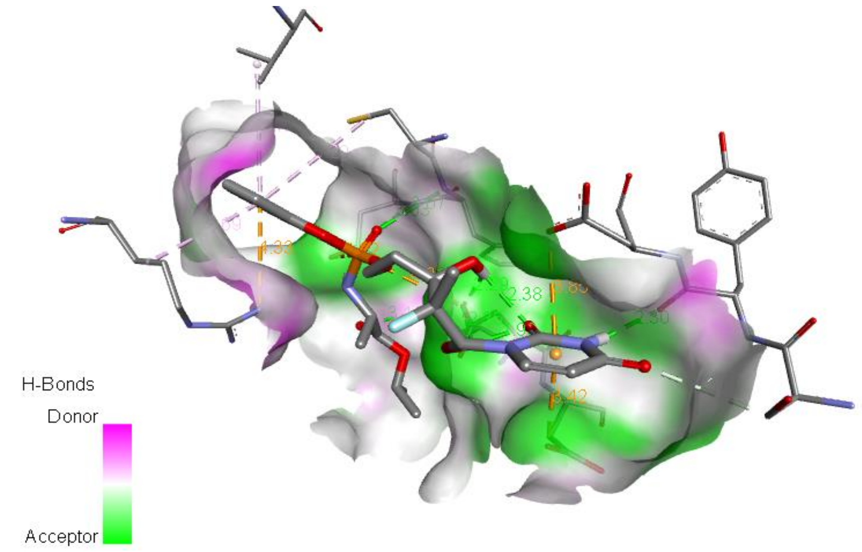


Figure 4.33 Showing Interaction of Sofosbuvir in binding pocket of NS5B polymerase (3D image)

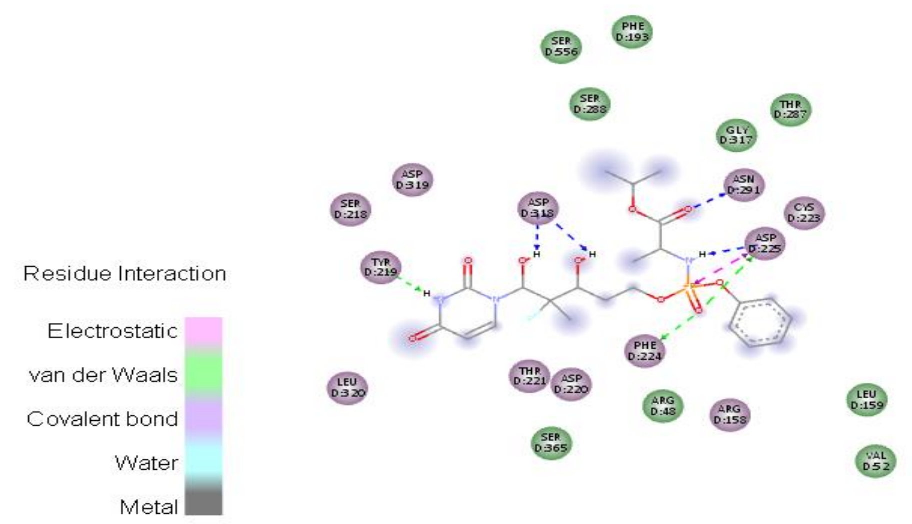


Figure 4.34 Showing Interaction of Sofosbuvir in binding pocket of NS5B polymerase (3D image)

Chapter 5

Discussion

Traditional healthcare system largely relies on plant derived medicines or phytochemicals. Biological activities of phytochemicals have been well documented. Antimicrobial, anti-cancer, anti-diabetic and many other biological activities of plants have been reported. Published data about the medicinal plants is increasing every day. Herbal medicines exhibit more effective actions and less harmful effects (Bent & Ko, 2004). Phytochemicals show low toxicity to mammalian systems and are easy to use. Published data about the medicinal plants is increasing every day.

In this study two plants i.e. *S. persica* and *S. oleoides* of genus *Salvadora* are studied for their potential biological activities. *S. persica* is known as "miswak" commonly and traditionally used as chewing stick. It is one of the plants which is cited in Holy Quran. WHO has promoted its use for oral hygiene. Both these plants contain a number of phytochemicals which have therapeutic potential e.g. salvadorin, beta sitisterol, salvadricine, salvado-urea, beta-isothiocyanate etc. (Kumar *et al.,* 2012; Sofrata *et al.,* 2012; Taha, 2008).

The objective of the study was to evaluate both plants for their therapeutic potentials. Leaves and roots were selected for extraction purposes. We used four solvents including ethanol, methanol, n-hexane and ethyl acetate for extraction from each part. Each extract was then evaluated for anti-oxidant capacity, cytotoxicity and antimicrobial activity. Potential antiviral activity was evaluated by *in silico* analysis.

Low levels of antioxidants are known to be the risk factor for the development of diseases. Antioxidants are therefore, important in prevention of diseases (Kalyanaraman, 2013). Antioxidant activity was evaluated by DPPH assay. The DPPH

assay revealed that the scavenging ability of the extracts increases with an increase in concentration of extracts. However, there was a significant difference between the scavenging abilities of ascorbic acid and extracts. There was non-significant difference between the free radical scavenging activities of different extracts. Both plants and extracts of their both parts showed good DPPH inhibition up to 60-65% at the concentration of 200µg. An ethanolic extract of *S. persica* showed DPPH inhibition up to 70% (Tiwari *et al.,* 2011). Fungal endophytic extracts of *S. oleoides* have been reported for antioxidant activity (Dhankar *et al.,* 2012). Ascorbic acid (standard) showed activity above 80%. So, it can be concluded that these plants have considerable antioxidant activity but not as significant as natural antioxidants e.g. ascorbic acid.

Extracts were evaluated for their cytotoxicity against human hepatocellular carcinoma (Huh-7) cells by using MTT assays in dose dependent manner. Generally, root extracts were safe i.e. showed very less inhibition in cell growth however, some leaf extracts showed cytotoxicity at concentrations approaching 250µg and higher. In case of *S. oleoides* no extract showed 50% reduction in the cell viability except ethanolic leaf extract, which reduced the cell viability up to 48%, 47% and 44% at the concentration of 250 µg, 500 µg and 1000 µg respectively.

In case of *S. persica*, no root extract showed 50% reduction in cell viability however, different leaf extracts reduced the cell viability below 50% at higher concentrations. Ethanolic extract reduced the cell viability up to 43% at the concentration of 1000 µg. Methanolic extract of the *S. persica* leaves was most lethal. It reduced the cell viability up to 28%, 39%, 44% and 46% at 1000 µg, 500 µg, 125 µg and 500 µg concentration respectively. In previous studies, polyamides isolated from *S. oleoides* showed weak activity against breast and colon cancer cell lines (Aroa *et al.,* 2014). Freshly cut *S. persica* showed no cytotoxic effects (Halawany, 2012).

Antibacterial activity of different extracts of *S.persica* and *S. oleoides* plants against three multi-drug resistant (MDR) strains of bacteria naming *Salmonella typhi*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* was evaluated. Present study indicated dose dependent bactericidal activity against these bacteria by showing zones of inhibition of increasing diameter with increasing concentration of extracts. Maximum diameter of zone of inhibition was found to be approximately 16 mm, signifying the efficacy of extracts against antibiotic resistant strains of bacteria. Minimum diameter of zone of inhibition was observed to be 6mm. There was no zone of inhibition around filter paper discs that were treated with de-ionized water, acting as negative control. So, it can be concluded that the zones of inhibition observed in discs treated with extracts, was actually due to presence of extracts. *S. oleoides* has been reported effective against *E. coli* and *S. aureus* (Suresh *et al.,* 2011). *S. persica* have been reported for antimicrobial activity against *Streptococcus mutans, P. aeruginosa, S. pyrogenis* and *S. faecalis* (Al-Sabawi *et al.,* 2007; Al-Bayati, & Sulaiman, 2008; Suffredini *et al.,* 2004). Miswak extracts have shown to reduce mouth bacterial plaques (Darout *et al.,* 2003). No study has been present for the anti-microbial activity against multidrug resistant strains of these plants. Current study investigated the bactericidal effects of these plants and obtained results indicated potent efficacy against (MDR) *Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella typhi*.

In silico analysis of some of the phytochemicals present in *Salvadora* plants revealed that these compounds have potential of antibacterial activities as well as anti-viral activities. In the present study, some bacterial multidrug resistance (MDR) causing proteins and 2 HCV proteins (NS3-4A, NS5B) were selected for molecular docking study. Proteins of multidrug resistance (MDR) used for docking were from the strains used in antibacterial study. 7-8 phytochemicals out of 11 candidates showed good

scores with respect to that of control drug used. However, SALVADORIN showed the most stable binding with all targets significantly. Salvadorin is a colorless amorphous powder with melting point of 161-163 ℃. It is a dimeric dihydroisocoumarin. It was first isolated from *S. oleoides* and its structure was isolated as 8-benzyl-6-[6-(6-ethyl-7-methyl-5,8-dihydro-2- naphthalenyl)-1-oxo-3,4-dihydro-1*H*-isochromen-8yl]-3, 4-dihyro-1*H*-isochromen-1-one by Mahmood and coworkers, 2012. So, we can propose that salvadorin is the potential candidate which can show both bactericidal and virucidal activities. Previously, it was proposed that β-isothiocyante is the potential phytochemical responsible for antibacterial and antiviral activities of *S. persica* (Halawany, 2012). Although we cannot establish it as a fact because β-isothiocyante has been shown to be the effective bactericidal by experimental data (Sofrata *et al.,* 2011). But in this study β-isothiocyante shown less stable binding as compared to that of salvadorin. There is only one study reporting for anti-HSV activity of *S. persica* extracts. Mechanism of anti-viral and bactericidal activities of *Salvadora* phytochemicals need to be explored by combining experimental, computational, purification and structural analysis.

In conclusion, extracts of leaves and roots of *Salvadora persica* and *Salvadora oleoides* were obtained by using 4 different solvents. These extracts were evaluated for their biological activity. Antioxidant ability was estimated by free radical scavenging activity against DPPH with considerable inhibition of the free radical in concentration dependent manner. Extracts were observed to have bactericidal ability against MDR *Staphylococcus aureus, Salmonella typhi* and *Pseudomonas aeruginosa*, implying their applications as anti-microbial agent and potential disinfectant. These extracts showed less cytotoxicity against HuH-7 cell line. A phytochemical salvadorin showed good

binding potential against some MDR and viral proteins. Their mechanisms of action yet to be determined experimentally.

Conclusion and recommendations

Current study was focused on evaluation of biological activity of *Salvadora persica* and *Salvadora oleoides.* After extraction biological activity was evaluated. These extracts were observed to have considerable antimicrobial feature and can be used as potential disinfectants. Antioxidant evaluation resulted in moderate free radical scavenging activity as compared to natural antioxidant, ascorbic acid. These extracts were found to have less cytotoxicity associated with them, which opens up a way to their use in therapeutics. Some selected phytochemicals shown to have antiviral and bactericidal activity against multi drug resistant strains in computational analysis.

After analyzing data and results depicted in this study, it is recommended to purify and characterize these extracts by using comprehensive techniques e.g. HPLC, GC-MS, NMR etc. and use *in vitro* techniques to find out the mechanisms underlying the bactericidal and virucidal activities of *Salvadora persica* and *Salvadora oleoides* which might help in discovering new drugs with broad therapeutic potentials.

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Appendix

Table A1. Showing the Descriptive statistics for DPPH inhibition by *S. oleoides* leaves

Table Analyzed	S.oleoides leaves				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	5				
F	14.43				
R squared	0.7937				
ANOVA Table	SS	df	MS		
Treatment (between columns)	15950	4	3988		
Residual (within columns)	4146	15	276.4		
Total	20100	19			
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Ethanol	54.24	4.614	Yes	**	15.61 to 92.87
Control vs Methanol	55.15	4.691	Yes	**	16.52 to 93.78

Control vs n-Hexane	75.39	6.413	Yes	***	36.76 to 114.0
Control vs Ethyl acetate	78.92	6.713	Yes	***	40.29 to 117.5
Ethanol vs Methanol	0.9092	0.07734	No	ns	-37.72 to 39.54
Ethanol vs n-Hexane	21.15	1.799	No	ns	-17.48 to 59.78
Ethanol vs Ethyl acetate	24.68	2.099	No	ns	-13.95 to 63.31
Methanol vs n-Hexane	20.24	1.722	No	ns	-18.39 to 58.87
Methanol vs Ethyl acetate	23.77	2.022	No	ns	-14.86 to 62.40
n-Hexane vs Ethyl acetate	3.524	0.2998	No	ns	-35.11 to 42.15

Table A2. Showing the Descriptive statistics for DPPH inhibition by *S. oleoides* roots

Table Analyzed	S. oleoides roots				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	5				
F	13.93				
R squared	0.7878				
ANOVA Table	SS	df	MS		

Treatment (between columns)	13450	4	3361		
Residual (within columns)	3621	15	241.4		
Total	17070	19			
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Ethanol	59.18	5.387	Yes	***	23.08 to 95.28
Control vs Methanol	64	5.825	Yes	***	27.90 to 100.1
Control vs n-Hexane	73.75	6.713	Yes	***	37.65 to 109.9
Control vs Ethyl acetate	54.17	4.931	Yes	**	18.07 to 90.27
Ethanol vs Methanol	4.819	0.4387	No	ns	-31.28 to 40.92
Ethanol vs n-Hexane	14.58	1.327	No	ns	-21.52 to 50.68
Ethanol vs Ethyl acetate	-5.006	0.4557	No	ns	-41.11 to 31.09
Methanol vs n-Hexane	9.757	0.8882	No	ns	-26.34 to 45.86
Methanol vs Ethyl acetate	-9.825	0.8943	No	ns	-45.92 to 26.27
n-Hexane vs Ethyl acetate	-19.58	1.783	No	ns	-55.68 to 16.52

Table A3. Showing the Descriptive statistics for DPPH inhibition by *S. persica* leaves

Table Analyzed	S.persica leaves				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	5				
F	40.63				
R squared	0.9155				
ANOVA Table	SS	df	MS		
Treatment (between columns)	17780	4	4445		
Residual (within columns)	1641	15	109.4		
Total	19420	19			

Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Ethanol	68.17	9.217	Yes	***	43.87 to 92.47
Control vs Methanol	73.45	9.931	Yes	***	49.15 to 97.75
Control vs n-Hexane	74.26	10.04	Yes	***	49.96 to 98.56
Control vs Ethyl acetate	79.94	10.81	Yes	***	55.64 to 104.2
Ethanol vs Methanol	5.279	0.7138	No	Ns	-19.02 to 29.58
Ethanol vs n-Hexane	6.088	0.8232	No	ns	-18.21 to 30.39

Table A4. Showing the Descriptive statistics for DPPH inhibition by *S. persica* roots

Table Analyzed	S.persica roots		
One-way analysis of variance			
P value	0.0007		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	5		
F	8.868		
R squared	0.7028		
ANOVA Table	SS	df	MS
Treatment (between columns)	12770	4	3193
Residual (within columns)	5401	15	360.1
Total	18170	19	

Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Ethanol	47.29	3.524	Yes	*	3.197 to 91.38
Control vs Methanol	51.44	3.833	Yes	*	7.342 to 95.53
Control vs n-Hexane	78.5	5.85	Yes	***	34.41 to 122.6
Control vs Ethyl acetate	42.96	3.201	No	ns	-1.138 to 87.05
Ethanol vs Methanol	4.145	0.3089	No	ns	-39.95 to 48.24
Ethanol vs n-Hexane	31.21	2.326	No	ns	-12.88 to 75.30

Table A5. Showing the Descriptive statistics for MTT assay by *S. oleoides* leaves

Table Analyzed	S. oleoides leaves				
One-way analysis of variance					
P value	0.0003				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	8.956				
R squared	0.4897				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	1.761				
P value	0.6234				

P value summary	ns				
Do the variances differ signif. (P < 0.05)	No				
ANOVA Table	SS	df	MS		
Treatment (between columns)	12700	3	4232		
Residual (within columns)	13230	28	472.6		
Total	25930	31			
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Ethanol vs Metahnol	-43.17	3.971	Yes	**	-74.02 to -12.31
Ethanol vs n-Hexane	-41.72	3.838	Yes	**	-72.58 to -10.86

Ethanol vs Ethyl acetate	-50.95	4.688	Yes	***	-81.81 to -20.10
Metahnol vs n-Hexane	1.443	0.1328	No	ns	-29.41 to 32.30
Metahnol vs Ethyl acetate	-7.789	0.7166	No	ns	-38.65 to 23.07
n-Hexane vs Ethyl acetate	-9.232	0.8494	No	ns	-40.09 to 21.63

Table A6. Showing the Descriptive statistics for MTT assay by *S. oleoides* roots

Table Analyzed	S. oleoides roots				
One-way analysis of variance					
P value	0.038				
P value summary	*				
Are means signif. different? (P < 0.05)	Yes				

Number of groups	4		
F	3.213		
R squared	0.2561		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	1.2		
P value	0.7529		
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	3304	3	1101
Residual (within columns)	9597	28	342.7
Total	12900	31	

Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Ethanol vs Metahnol	-27.49	2.969	Yes	*	-53.76 to -1.206
Ethanol vs n-Hexane	-8.963	0.9682	No	ns	-35.24 to 17.32
Ethanol vs Ethyl acetate	-6.985	0.7546	No	ns	-33.26 to 19.29
Metahnol vs n-Hexane	18.52	2.001	No	ns	-7.757 to 44.80
Metahnol vs Ethyl acetate	20.5	2.215	No	ns	-5.780 to 46.78
n-Hexane vs Ethyl acetate	1.977	0.2136	No	ns	-24.30 to 28.26

Table A7. Showing the Descriptive statistics for MTT assay by *S. persica leaves*

Table Analyzed	S. persica leaves
One-way analysis of variance	
P value	< 0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	4
F	11.84
R squared	0.5593
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	4.643

P value	0.1999				
P value summary	ns				
Do the variances differ signif. (P < 0.05)	No				
ANOVA Table	SS	df	MS		
Treatment (between columns)	22800	3	7601		
Residual (within columns)	17970	28	641.7		
Total	40770	31			
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Ethanol vs Metahnol	26.95	2.127	No	ns	-9.012 to 62.90
Ethanol vs n-Hexane	-34.85	2.751	No	ns	-70.81 to 1.110

Ethanol vs Ethyl acetate	-37.86	2.989	Yes	*	-73.82 to - 1.900
Metahnol vs n-Hexane	-61.79	4.879	Yes	***	-97.75 to - 25.84
Metahnol vs Ethyl acetate	-64.8	5.116	Yes	***	-100.8 to - 28.85
n-Hexane vs Ethyl acetate	-3.01	0.2376	No	ns	-38.97 to 32.95

Table A8. Showing the Descriptive statistics for MTT assay by *S. persica* roots

Table Analyzed	S. persica roots				
One-way analysis of variance					
P value	0.4374				
P value summary	ns				

Are means signif. different? (P < 0.05)	No				
Number of groups	4				
F	0.9338				
R squared	0.09095				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	3.296				
P value	0.3482				
P value summary	ns				
Do the variances differ signif. (P < 0.05)	No				
ANOVA Table	SS	df	MS		
Treatment (between columns)	1324	3	441.2		

Residual (within columns)	13230	28	472.5		
Total	14550	31			
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Ethanol vs Metahnol	-16	1.472	No	ns	-46.85 to 14.86
Ethanol vs n-Hexane	-2.702	0.2487	No	ns	-33.56 to 28.15
Ethanol vs Ethyl acetate	-11.24	1.034	No	ns	-42.09 to 19.62
Metahnol vs n-Hexane	13.29	1.223	No	ns	-17.56 to 44.15
Metahnol vs Ethyl acetate	4.758	0.4378	No	ns	-26.10 to 35.61
n-Hexane vs Ethyl acetate	-8.536	0.7854	No	ns	-39.39 to 22.32

Table A9. Showing the data generated by Antibacterial Assay

S.persica leaves						S.persica roots				
Salmonella typhi						Salmonella typhi				
zone of inhibition						zone of inhibition				
Conc.	Ethanol	methanol	n-Hexane	ethyl acetate		Conc.	Ethanol	methanol	n-Hexane	ethyl acetate
50µg	8	7	9	8		50µg	9	7	6	7
100µg	9	8	9	8		100µg	9	8	7	7
150µg	9	8	10	10		150µg	11	8	7	9
200µg	10	9	10	10		200µg	11	10	8	9
250µg	11	10	11	12		250µg	12	12	9	11
300µg	11	12	12	13		300µg	13	12	10	11
S.persica leaves						S.persica roots				
Staphylococcus aureus						Staphylococcus aureus				
zone of inhibition						zone of inhibition				
Conc.	Ethanol	methanol	n-Hexane	ethyl acetate		Conc.	Ethanol	methanol	n-Hexane	ethyl acetate
50µg	7	6	7	6		50µg	6	6	7	8
100µg	8	6	8	6		100µg	6	7	9	8
150µg	8	7	8	6		150µg	7	7	9	8

200μg	8	7	8	8		200μg	7	8	9	9
250μg	9	8	8	8		250μg	8	8	10	9
300μg	9	8	9	8		300μg	8	8	10	10
	9	8	9	8						
S.persica leaves						S.persica roots				
Pseudomonas aeruginosa						Pseudomonas aeruginosa				
zone of inhibition						zone of inhibition				
Conc.	Ethanol	methanol	n-Hexane	ethyl acetate		Conc.	Ethanol	methanol	n-Hexane	ethyl acetate
50μg	8	7	7	8		50μg	8	9	8	8
100μg	9	8	8	8		100μg	10	9	8	8
150μg	11	8	8	10		150μg	12	10	9	8
200μg	11	10	9	10		200μg	13	11	10	9
250μg	13	11	9	12		250μg	14	11	10	10
300μg	14	11	9	12		300μg	15	14	11	10

S.Oleoides leaves						S.Oleoides roots				
Salmonella typhi						Salmonella typhi				
zone of inhibition						zone of inhibition				
Conc.	Ethanol	methanol	n-Hexane	ethyl acetate		Conc.	Ethanol	methanol	n-Hexane	ethyl acetate

50µg	8	8	8	8		50µg	9	7	6	8
100µg	9	8	8	8		100µg	9	8	7	9
150µg	10	9	10	9		150µg	9	8	7	9
200µg	11	10	10	9		200µg	11	9	8	10
250µg	11	10	11	11		250µg	13	11	9	11
300µg	11	11	11	13		300µg	14	12	10	13
S.Oleoides leaves						S.Oleoides roots				
Staphylococcus aureus						Staphylococcus aureus				
zone of inhibition						zone of inhibition				
Conc.	Ethanol	methanol	n-Hexane	ethyl acetate		Conc.	Ethanol	methanol	n-Hexane	ethyl acetate
50µg	8	7	7	6		50µg	8	9	8	7
100µg	9	8	7	7		100µg	8	9	8	9
150µg	9	10	8	7		150µg	10	10	8	11
200µg	10	10	10	8		200µg	11	10	10	13
250µg	10	12	13	8		250µg	13	11	11	15
300µg	12	13	14	9		300µg	15	12	11	16
S.Oleoides leaves						S.Oleoides roots				
Pseudomonas aeruginosa						Pseudomonas aeruginosa				

zone of inhibition						zone of inhibition				
Conc.	Ethanol	methanol	n-Hexane	ethyl acetate		Conc.	Ethanol	methanol	n-Hexane	ethyl acetate
50µg	8	7	7	8		50µg	8	7	6	6
100µg	8	7	8	8		100µg	9	7	6	7
150µg	8	8	8	9		150µg	9	8	7	8
200µg	9	8	10	10		200µg	9	8	8	8
250µg	9	9	10	12		250µg	10	8	8	10
300µg	10	9	11	12		300µg	12	9	9	10

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