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Full Length Research Paper

Biological screening of *Hedera nepalensis*

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The aim of the current work was to screen the crude methanolic extract (Cr. MeOH Ext.) and various fractions of *Hedera nepalensis* for antibacterial, antifungal, brine-shrimp lethality, phytotoxicity, heamagglutination, insecticidal and nitric oxide free radical scavenging assay. The results revealed that chloroform ($CHCl_3$) fraction of the plant possess good antibacterial activity (60%) against *Staphylococcus aureus* while the rest of the fractions and Cr. MeOH Ext. showed moderate (>40%) and low antibacterial activity (<40%). The Cr. MeOH Ext., *n*-hexane and ethyl acetate (EtOAc) fractions of the plant showed good phytotoxicity at higher concentration (1000 µg/ml) while low phytotoxicity was observed at lower concentrations (100 and 10 µg/ml), against *Lamina minor*. The Cr. MeOH Ext., *n*-hexane and $CHCl_3$ fractions of the plant showed good nitric oxide free radical scavenging activity at a concentration of 1.5 mg/ml. Moderate brine shrimp lethality was also observed for Cr. MeOH Ext. and $CHCl_3$ fraction at a concentration of 1000 µg/ml, against *Artemisia salina*. The plant materials (Cr. MeOH Ext. and various fractions) exhibited no antifungal, insecticidal or heamagglutination activity.

Key words: Antibacterial, antifungal, brine-shrimp lethality, phytotoxicity, heamagglutination, insecticidal and nitric oxide free radical scavenging assay.

INTRODUCTION

Plants are employed to be used as medicines since long but specifying them for diseases started later on. Various efforts were made in the past to explore the medicinal flora and the pharmacists are still trying their best to explore the importance of medicinal flora throughout the world. Pakistan is the country with four seasons a year and is richly accumulated with phyto-medicinal flora that needs extensive labor and effort to be explored. It has been estimated that approximately 8000 plant species have medicinal importance of which 2000 are found in Pakistan (Oliyiowola, 1984).

In South Asian countries, the dependence on traditional use of herbs get more important as in these countries the primary health care is less affordable as compared to the traditional herbal medication (Zhang, 2002). In continuation to the previous work done, by our group, on medicinal plants, the current study was carried out on *Hedera nepalensis* (Himalayan Ivy) which belongs to family Araliaceae. The family is native to Nepal and Bhutan as well as China, Afghanistan, India, Laos Thailand, Myanmar and Vietnam at altitude of approximately 1000 to 3000 m. The family is represented here by 3 genera, each having a single species (Shahina, 1989; Nasir, 1975).

Leaves of *H. nepalensis* are used traditionally for treatment of diabetes (Frohne and Pfander, 2004). *H. nepalensis* is an important food supplement for animals and local inhabitants of study area and it also have some other profitable uses such as medicinal plants, essentials, fibers and dyes resources. Various parts of the nettle plant can be used as food, medicines, fibers, cosmetics, industry and biodynamic agriculture and is also very

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significant plant for wildlife (Meyer et al., 1993). Extracts obtained from the leaves of *H. helix* are often used in the treatment of upper respiratory tract conditions and inflammatory bronchial disease. Clinical improvements in lung function have been observed, on the basis of spirometric and plethysmographic measurements, as well as for the accompanying signs of coughing and expectoration (Gulyas et al., 1997; Mansfeld et al., 1997, 1998). Leaves of the plant are used to treat cancer (Hamayun et al., 2006).

Approximately 10% of the ethanolic ivy extract consists of a complex mixture of saponins with the bis-desmoside hederacoside C as the main component, together with smaller amounts of the monodesmoside R-hederin and the aglycon hederagenin. The expectorant activity of saponins is thought to be mediated by the gastric mucosa, with reflex stimulation of the bronchial mucous glands via parasympathic pathways. This mode of action, however cannot explain the bronchiolytic effects, as shown by dose dependent inhibition of PAF-induced broncho-construction in guinea pigs by ethanolic extract of Ivy (Haen, 1996; Stahl, 1969) or by *in-vitro* inhibition of acetylcholine-induced spasm in guinea pig ileum by R-hederin and hederagenin (Trute et al., 1997). Moreover, Ivy leaf extracts are spasmolytic, reducing smooth muscle spasm, as well as bronchodilatory and anti-bacterial, which is mainly due to their triterpene saponin content (Cioaca et al., 1978; Trute et al., 1997; Bedir et al., 2000). The present manuscript deals with screening of Cr. MeOH Ext. of the plant for various biological activities.

MATERIALS AND METHODS

Plant material

Plant material aerial parts were collected from Bara Gali, KPK, Pakistan, in December 2009 to January 2010 and was identified by Prof. Dr. Abdur Rashid, Plant Taxonomist, Department of Botany, University of Peshawar, KPK, Pakistan.

Extraction

Plant material was shade dried, chopped and grinded to fine powder with the help of electric grinder. The plant material (6 kg) was then soaked in marketable grade MeOH for 15 days room temperature. After 15 days of soaking, MeOH soluble material was then filtered off and all these left over filtrates were mixed and concentrated under vacuum below 40°C. At the end a blackish Cr. MeOH Ext. (915 g) was obtained.

Fractionation

A suspension of Cr. MeOH Ext. (850 g) was made in 400 ml of a distilled water and fractionated with *n*-hexane (3 x 400 ml), $CHCl_3$ (3 x 400 ml), EtOAc (3 × 400 ml). Different fractions; *n*-hexane (100 g), $CHCl_3$ (80 g), EtOAc (140 g) and aqueous (155 g) were obtained after fractionation. Approximately, 65 g of the Cr. MeOH Ext. was kept for other biological screenings.

Anti-bacterial activity

For the cure of various infections several decoctions of plants materials are used in medicine that gives beneficiary results in treatments. The screening against diverse pathogens was carried out while keeping in view their clinical importance. The antibacterial activity was performed as per our reported procedure (Bashir et al., 2011). Test organisms used in the experiment includes; *Escherichia coli, Staphylococcus epidermidis, Salmonella typhi, Streptococcus pneumonia, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus pumilus*.

Nutrient broth was prepared and the test organisms were then inoculated and incubated at 37°C for 24 h. In sterile Petri dishes, the autoclaved nutrient agar medium was transferred and was allowed to cool. When the plates were hardened and solidified, they were shifted to the incubator in order to check for sterility for 24 h at 37°C. After sterility check, the sterile plates were used for inoculation of test pathogens. Minute volume form the nutrient broth containing the culture was spread over plates and left for 10 min.

By the help of a 6 mm borer, wells were made in nutrient agar plates. The test samples were made ready by dissolving 3 mg of the sample in 1 ml DMSO. 100 µl of the test samples were then introduced into the wells by using micropipette. The standard drug employed was Imipenem. DMSO was used as a negative control. The plates were then allowed undisturbed for 2 h so that the samples cam diffuse well into the media. The plates were then incubated for 24 h at 37°C. The zones of inhibition were measured after incubation time. The percent (%) inhibition was calculated by the following formula:

Percentage inhibition=Zone of inhibition of sample/zone of inhibition of standard × 100

Antifungal activity

Test organisms

Various pathogenic fungal species were used to perform antifungal activity; *Aspergillus niger, Aspergillus flavus, Penicillium notatum, Fusarium oxysporum, Trichoderma harzianum and Rhizopus stolonifer.* The activity was performed according to the procedure of Bashir et al. (2011). Stock solutions of the test samples (24 mg/ml) were prepared in sterile DMSO. Sabouraud dextrose agar (SDA) medium was prepared and 4ml aliquots were transferred to sterilized test tubes. At slant positions the test tubes were allowed to cool and solidify. The test tubes were streaked with fresh culture of test fungi then inoculated. The standard drug Amphotericin-B served as positive control whereas DMSO served as negative control. The test tubes were then incubated for 7 days at 25°C. The linear growth of the fungi was measured on the 7^{th} day. Percent (%) inhibition was computed by the formula given as follows:

Percentage inhibition = 100-(Growth in sample/Growth in negative control) × 100.

Phototoxic activity

The test samples were screened for phytotoxicity against *Lemnaminor*, as per reported procedure of McLaughlin (1991). The stock solution was prepared in MeOH at concentration of 3 mg/ml and 1000, 100 and 10 µg/ml was transferred to separate flasks. The flasks were left overnight so that the MeOH can evaporate. After the

evaporation of MeOH, 20 ml of E-media was introduced in each flask and fifteen healthy plants were introduced into each flask. The flasks were incubated for 7 days at a 28 ± 1°C using a growth cabinet The growth regulation was measured in percentage (%) with respect to the negative control on day 7th by formula given as follows:

Percent growth regulation = 100-(Number of fronds in test/Number of fronds in control) × 100

Insecticidal assay

Contact method was used for the determination of the insecticidal activity of the Cr. MeOH Ext. and other fractions of *H. nepalensis* (Ahn et al., 1995). The contact toxicity array was performed by the following method: In volatile organic solvent, an appropriate quantity (100, 500, 100, 50, 10 ppm) of the test samples was dissolved. The contents were then covered on the inner surface of 20 ml glass upon vials evaporation. Until the test solution was circulated in the vial inner wall and the floor, each vial glass was turned around by hand and the solvent has mostly evaporated. In a fume hood, each glass vial is kept for about 10 min to completely evaporate the remaining organicsolvent. Care should be taken to homogeneouslycoat on the inner surface of the vial with test sample(s). Five test insects were kept in each vial with adequate food. The standard drug, Permethrin, at concentration 235 µg/cm^2 and respective solvent was used as positive and negative controls, respectively. The results were recorded after 24 h for 5 days.

Brine shrimp lethality bioassay

A. salina (brine-shrimp eggs) was used to determine the cytotoxicity of the plant materials as per our reported procedure (Bashir et al., 2009). A thin rectangular plastic dish 22x32 cm size was taken having sea water, made by the help of commercial salt blend containing; instant Ocean, Aquarium System, Inc., Mentor, OH, USA along with double distilled water. A perforated device was used for unequal partition in the plastic dish. To the larger compartment, which was darkened, eggs of about 50 mg were sparged. The smaller compartment of the plastic dish was kept open to ordinary light. This whole system was kept at room temperature for 48 h and the eggs were allowed hatch and mature.Then using a Pasteur pipette the nauplii were collected from the lighten side. From the stock solution (20 mg/2 ml), 5, 50 and 500 µl were transferred into vials (3 vials/concentration) with final concentration of 10, 100 and 1000 µg/ml. The vials were then allowed over-night or placed in the hood for half an hour to evaporate the organic solvents completely. 1 ml of the sea water was then poured to each vial and 10 larvae per vial were placed with the help of Pasteur pipette. The final volume of each vial was adjusted to 5 ml with sea water. The vials were incubated under illumination at 26±1ºC for 24 h. Other vials were supplemented with respective organic solvent as negative control and reference cytotoxic drug as positive control. After incubation period the survived shrimps were counted using a magnifying glass.

Heamagglutination activity

Haemagglutination activity of test samples was performed according to procedure of Naqvi et al. (1998). Phosphate buffer (pH 7.0) was prepared by dissolving 0.453g of KH_2PO_4 and 0.47g ofNa_2HPO_4, each in 50 ml of distilled water. The dissolved KH_2PO_4 and Na_2HPO_4 were mixed in ratio of 3:7 (V/V). Stock solution (1 mg/ml) of the test samples was prepared in DMSO and different dilutions; 1:2, 1:4, 1:8 and 1:16 were made in phosphate buffer. Fresh blood were collected from healthy persons on the same day of the experiment and centrifuged, the plasma was discarded and red blood cells (RBC's) were collected for the next steps. 2% RBC's suspension was prepared in the phosphate buffer. From each dilution, 1 ml of sample was taken in a test tube and then adds 1 ml of the RBC's suspension to the sample. Incubate the test tubes for 30 min at 37°C. After incubation, the test tubes were examined for the button formation, rough button formation indicates positive and smooth button formation indicates negative results.

Nitric oxide free radical scavenging assay

At physiological pH, the sodium nitroprusside when in aqueous solution impulsively produces nitric oxide, to produce nitrite ion. These nitrite ions on reaction with sulphanilic acid produce a *p*-diazonium salt. Napthyl ethylenediamine dihydrochloride (0.1% w/v) on reaction with *p*- diazonium salt, form a pink complex of Azo dye and the absorbance rate of the pink chromophore is calculated at 570 nm.

The NO free radical scavenging activity was carried out as per reported procedure of Bashir et al. (2011). Stock solutions (3 mg/ml) of the test samples were prepared in DMSO. From this stock solution, by dilution, different concentration of the test samples; 0.3, 0.6, 0.9, 1.2 and 1.5 mg/ml were prepared. 10 µl from each concentration of the test sample were taken in microtiter plate and add 20 µl of phosphate buffer, then 70 µl of sodium Nitroprusside was added. The microtiter plate was incubated at 20 to 25°C for one and half hour. The plate was swirled well after incubation and 50 µl of sulphanilic acid was added. Absorbance (pre-absorbance) was taken at 570 nm and then 50 µl of [N-(1-Napthyl) Ethylenediaminedi-hydrochloride] was added, swirled well again and then the final reading was taken. Vitamin C and DMSO were run as positive control and blank, respectively.

RESULTS AND DISCUSSION

Antibacterial activity

Currently, antibiotic resistance is one of the major problems faced by medical science and finding new and innovative antimicrobials will help to tackle this problem (Freeman, 1997). *S. aureus* has become resistant to several antibiotics to which it was previously susceptible these includes; tetracycline, penicillin G, macrolides, gentamicin and lincosamides (Ayliffe, 1997).

The test samples were tested against the selected pathogens and the results obtained are presented in Figure 1. The Cr. MeOH Ext. showed moderate activity against *E. coli* (47.8%), *S. pneumoniae (*46.1%), *S. aureus (*48%), *P. aeruginosa* (40%) and *B. pumilus* (41.9%). Low antibacterial activity was shown by the Cr. MeOH Ext. against *S. epidermidis* (36.6%) and *S. typhi* (37.8%). The *n*-hexane fraction exhibited moderate activity of 50, 48, 42 and 48.3% against *E. coli, S. aureus*, *P. aeruginosa* and *B. pumilus*, respectively. Low antibacterial activity was shown by this fraction against *S. epidermidis* (39.3%), *S. typhi* (35.7%) and *S. pneumoniae* (*3*4.6%).

The $CHCl_3$ fraction showed good activity against *S.*

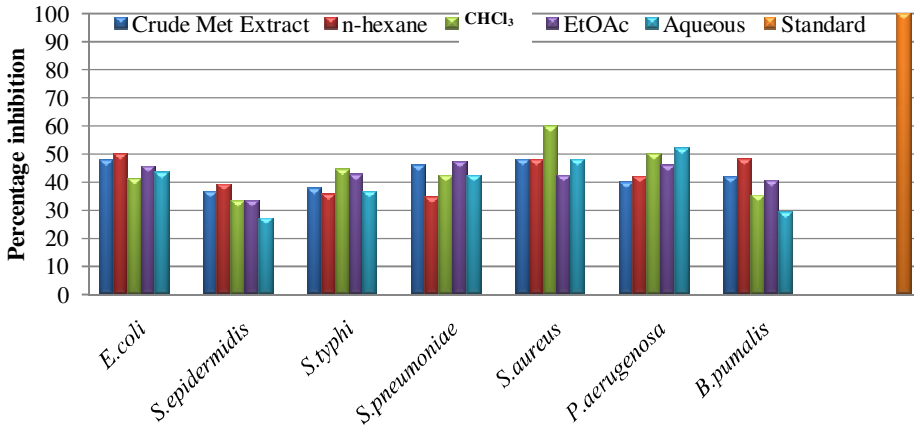

Figure 1. Antibacterial activity of Cr. Met. Ext. and various fractions of *H. nepalensis.*

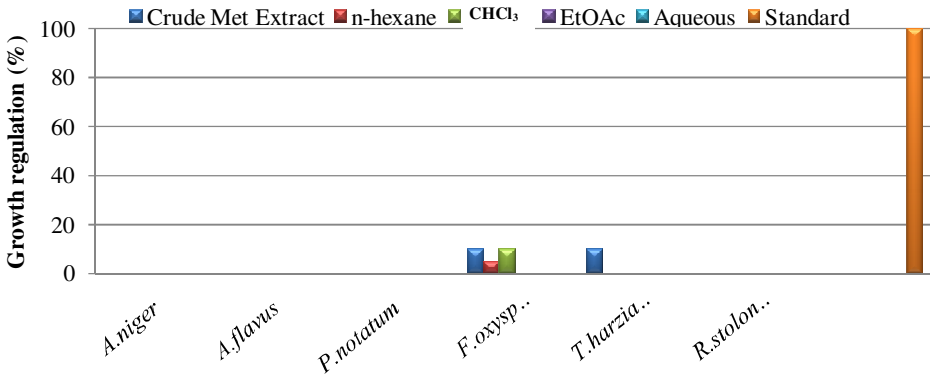

Figure 2. Antifungal activity of Cr. Met. Ext. and various fractions of *H. nepalensis.*

*aureus (*60%), moderate activity against *E. coli* (41.3%), *S. typhi* (44.6%), *S. pneumoniae (*42.3%), *P. aeruginosa* (50%) and low activity against *S. epidermidis* (33.3%) and *B. pumilus* (35.4%). The EtOAc fraction exhibited moderate activity of 45.6, 42.8, 47.3, 42.4, 46 and 40.6% against *E. coli, S. typhi*, *S. aureus*, *S. pneumoniae*, *P. aeruginosa* and *B. pumilus*, respectively. Low antibacterial activity was shown by this fraction against *S. epidermidis* (33.3%). The aqueous fraction exhibited moderate activity against *E. coli* (43.4%), *S. pneumoniae* (42.3%), *S. aureus* (48%) and *P. aeruginosa* (52%). Low antibacterial activity was shown by this fraction against *S. epidermidis* (27.2%), *S. typhi* (36.7%) and *B. pumilus* (29.3%).

The previous results indicate that $CHCl_3$ fraction have good activity against *S. aureus* so, this fraction of the plant can be further used for isolation of the active constituent(s).

Antifungal activity

The test samples were screened for their antifungal activity against *A.niger*, *A. flavus*, *P. notatum*, *F. oxysporum*, *T. harzianum* and *R. stolonifer*. The results are given in Figure 2. Amphotericin-B served as standard drug. Against *F. oxysporum* percent growth regulation was: Cr. MeOH Ext. (10%), *n*-hexane (5%) and chloroform (10%). The EtOAc and aqueous fractions were inactive against *F. oxysporum*. The Cr. MeOH Ext. exhibited low activity (10%) against *T. harzianum* while rests of the fractions were inactive against it. All the test sensible. *L. minor* L., a small aquatic monocot, is used as a model system to detect phytotoxic compounds because of their sensitivity to bioactive compounds, natural anti-tumor agents and detection of new plant growth stimulant (Karmen et al., 2003). The results of phytotoxic activity of the test samples using *Lemna* assay are presented in Figure 3. The Cr. MeOH Ext. showed growth regulation of 46.66, 33.33 and 13.33% at 1000 and 100 and 10 µg/ml, respectively. The percent growth regulation of *n*-hexane fraction was 53.33, 33.33 and 6.66 at 1000 and 100 and 10 µg/ml, respectively. The percent growth regulation of $CHCl_3$ fraction was 26.66, 13.33 and 13.33%, EtOAc fraction was 60, 33.33 and 13.33% and aqueous fraction

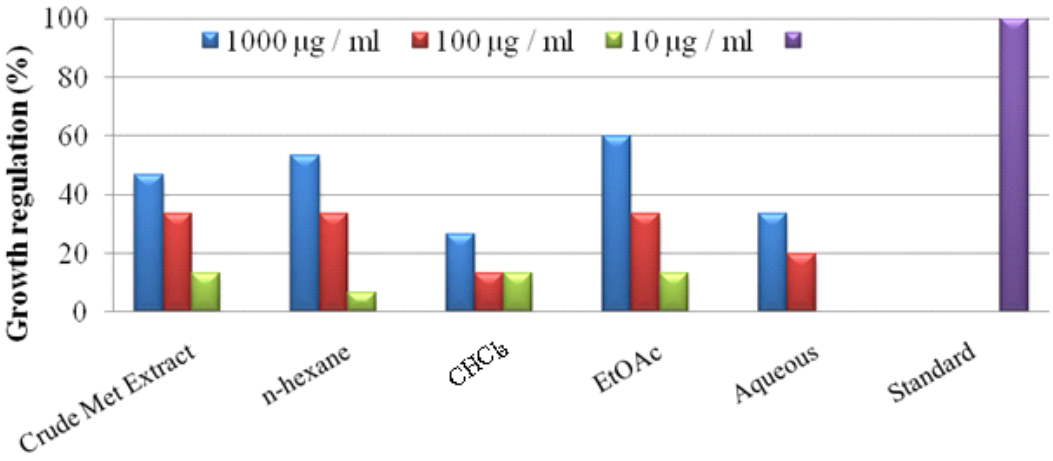

Figure 3. Phytotoxic activity of the Cr. Met. Ext. and various fractions of *H. nepalensis.*

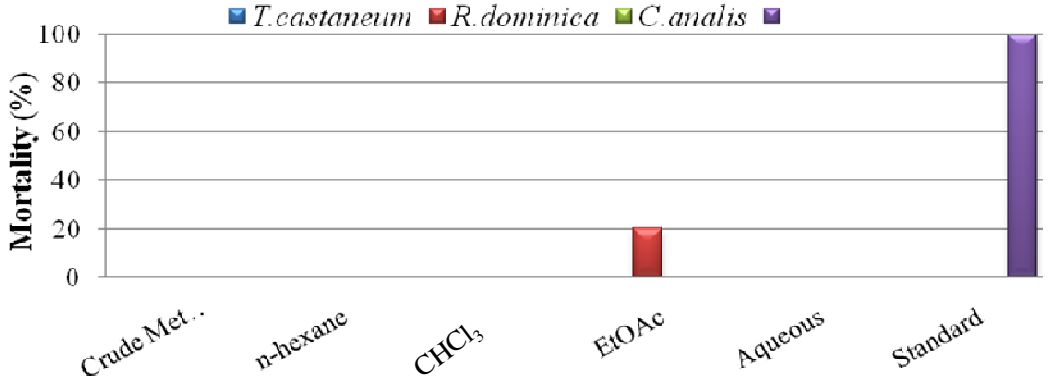

Figure 4. Insecticidal activity of Cr. Met. Ext. and various fractions of *H. nepalensis*.

was 33.33, 20 and 0% at 1000 and 100 and 10 µg/ml respectively. The previous results shows that the EtOAc The synthetic insecticides are harmful both for humans and environment due to their toxic effect. Therefore these harmful insecticides should be replaced by environment friendly insecticides from the natural resources (Suszkiw, 1998). The insecticidal activity of test samples was carried out against *T. castaneum, R.* dominica and *C. analis*. The results are mentioned in Figure 4. Only the EtOAc fraction showed low activity of 20% against *R. dominica*. The other entire test samples that is, Cr. MeOH Ext. and various fractions were inactive against all of the test organisms.

Haemagglutination activity lectin specificities have been used to study the sugar components on cancerous and normal cell surfaces (Lis and Sharon, 1986) and structural and functional roles of cell surface carbohydrates (Sharon and Lis, 1972). Agglutinin from the plant sources is advantageous over animal sources because they are available in large quantities and are economical.

The results of the haemagglutination activity of the test samples were determined against human RBC's of all blood groups. The results are shown in Table 1. All of the test samples were inactive against RBC's of all blood groups. They do not showed any agglutination which means that *H. nepalensis* lacks phytolectins.

Nitric oxide (NO) free radical scavenging assay

An important messenger molecule in many pathological and physiological processes within the mammalian body is Nitric oxide (NO). Nitric oxide has both beneficial and detrimental effect on the human health (Aldrich Library, 1992). NO when produced in appropriate amount helps in the protection of organs for example liver from ischemic damage. A higher level of NO is toxic to tissue and contributes too many diseases like carcinomas, multiple scleroses (Dari et al., 1991). Keeping in view the

Table 1. Haemagglutination activity of the Cr. MeOH Ext. and various fractions of *H. nepalensis*.

Blood group	Cr. Met. Ext.				*n*-hexane				Chloroform				Ethyl acetate				Aqueous			
	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
AB^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AB^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2. NO free radical scavenging assay of the Cr. Met. Ext. and fractions of *H. nepalensis*.

Concentration of sample (mg/ml)	Percent activity				
	Cr. Met. Ext	*n*-hexane	$CHCl_3$	EtOAc	Aqueous
0.3	18.52	23.10	24.96	0	0
0.6	25.66	24.58	28.11	2.44	0
0.9	36.31	27.22	36.27	8.84	2.4
1.2	48.47	37.63	40.90	17.96	4.9
1.5	54.72	41.08	47.54	23.92	12.33

Standard: Vitamin C was used as a standard at concentration of 47.87 µg/ml.

importance of NO, the test samples were screened for NO free radical scavenging at different concentrations that is, 0.3. 0.6, 0.9, 1.2 and 1.5 mg/ml. Table 2 represent the results. At 0.3 mg/ml, the $CHCl_3$, *n*-hexane fractions and Cr. MeOH Ext. exhibited low activity of 24.96, 23.10 and 18.52%, respectively. The EtOAc and aqueous fraction were inactive at this concentration.

Results of the assay at 0.6 mg/ml showed that the Cr. MeOH Ext., *n*- hexane and $CHCl_3$ fractions have 25.66, 24.58 and 28.11% NO free radical scavenging activity, respectively. The EtOAc fraction showed low activity of 2.44% while aqueous fraction was inactive at this concentration. The Cr. MeOH Ext., *n*- hexane, $CHCl_3$, EtOAc and aqueous fractions showed NO free radical scavenging activity of 36.31, 27.22, 36.27, 8.84 and 2.4%, respectively at 0.9 mg/ml. At 1.2 mg/ml, the results recorded showed that the NO free radical scavenging activity of Cr. MeOH Ext., *n*-hexane, $CHCl_3$, EtOAc and aqueous fractions were 48.47, 37.63, 40.90, 17.96 and 4.9%, respectively. At a concentration of 1.5 mg/ml, NO free radical scavenging activity was; Cr. MeOH Ext. (54.72%), *n*-hexane (41.08%), $CHCl_3$ (47.54%), EtOAc (23.92%) and aqueous fraction (12.33%), respectively.

The previous results indicate that the Cr. MeOH Ext., *n*-hexane and $CHCl_3$ fractions of *H. nepalensis* have concentration dependent NO free radical scavenging activity. Therefore, this plant can be searched for free radical scavenging

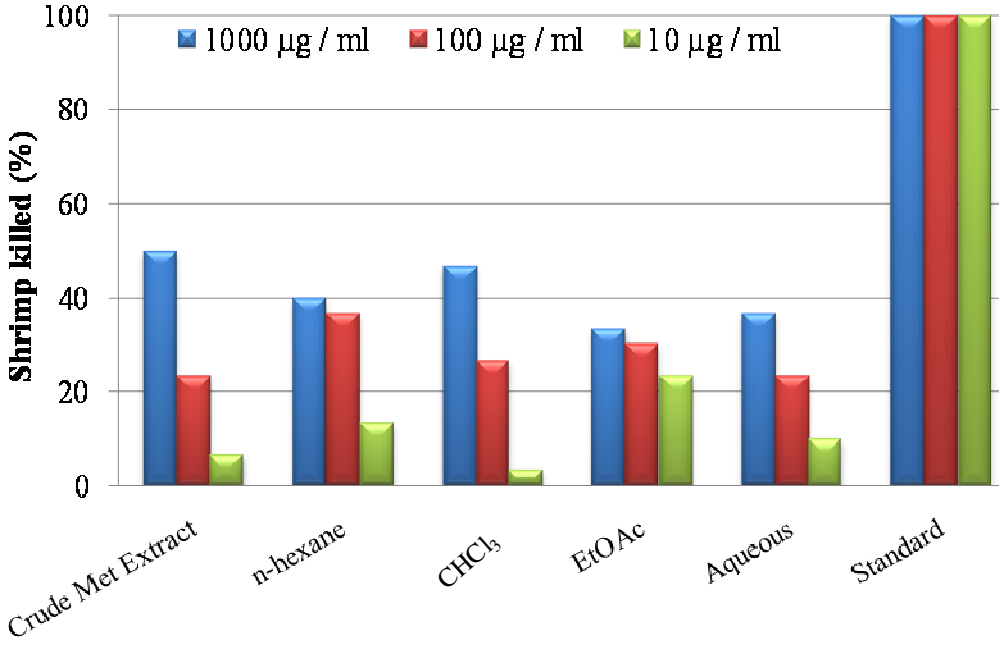

Figure 5. Brine shrimp cytotoxicity of Cr. Met. Ext. and various fractions of *H. nepalensis* standard drug: Etopsoide at concentration of 7.4625 µg/ml.

compounds.

Brine shrimp lethality bioassay

Brine shrimplethality bioassay was used to check the cytotoxic effect of the test samples. The results of the assay are given in Figure 5. The results indicated that the Cr. MeOH Ext. showed good cytotoxicity (50%) at 1000 µg/ml. At 100 and 10 µg/ml, cytotoxicity was 23.33 and 6.66% respectively. The *n*-hexane fraction showed a moderate cytotoxicity (40%) at 1000 µg/ml and low activity (36.66 and 13.33%) at concentration of100 and 10 µg/ml, respectively. The $CHCl_3$ fraction showed moderate brine shrimplethality (46.66%) at 1000 µg/ml. It showed low brine shrimplethality (26.66 and 3.33%) at 100 and 10 µg/ml, respectively. The EtOAc fraction showed 33.33, 30.33 and 23.33% cytotoxicity at 1000, 100 and 10 µg/ml, respectively. The cytotoxicity observed for aqueous fraction was 36.66% at 1000 µg/ml, 23.33% at 100 µg/ml and 10% at 10 µg/ml.

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