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In vitro antimicrobial and cytotoxic manifestations of *Dicliptera roxburghiana*

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Abstract: Emerging resistance in microorganisms is a growing threat to human beings due to its role in pathological manifestations in different infectious diseases. This study was designed to investigate the antimicrobial and cytotoxic potential of methanol extract of *Dicliptera roxburghiana* and all its derived fractions. Antibacterial (against six bacterial strains) and antifungal (against four fungal strains) activities were investigated by agar well diffusion method and agar slants method, respectively. Cytotoxicity assay was carried out by using Brine shrimps eggs. In antibacterial evaluation, MIC values and zone of inhibition were measured and were found very effective for DRME, DRHF, DRCF and DREF while these were moderate for DRBF and DRAF. For antifungal assay, DRME and DRHF were potently active and showed more than 70% fungal growth inhibition where as DRCF and DRBF were also displaying appreciable inhibition. Cytotoxic measurements were very good for DRME, DRHF and DRAF with LD_{50} values 215, 199 and 392µg/ml respectively. These results confirmed antimicrobial and cytotoxic potential of the plant and all its derived fractions. Hence it can be concluded that plant contain some important compounds that can be used as antimicrobial source for the treatment of different infectious disease.

Keywords: *D. roxburghiana*, antimicrobial, microbial resistance, cytotoxicity.

INTRODUCTION

Infectious diseases, of both bacterial and fungal origin, became foremost hazard for human beings. They are key threat to the population health mainly due to the vaccine unavailability or inadequate chemotherapy (Assob *et al.,* 2011). The growing tendency of microbial resistance to antibiotics has turn out to be of great concern all over the world (Gardam, 2000). Hence emerging bacterial resistance now a days led to a revival in follow a line of investigation to explore the antimicrobial role of herbs against challenging strains (Alviano and Alviano, 2009; Hemaiswarya *et al*., 2008).

With the growing threats of infectious diseases due to resistance there is intense need to unlock the secrets of herbal remedies. Many endeavors have been made to investigate new antimicrobial compounds from various folk medicinal plants and their fractions (Khan *et al.,* 2010). People are ever more engrossed in herbal medicine, as complementary and alternative medicines, because they observe these therapeutic agents as being safe, sound and effective as well (Wendakoon *et al*., 2012). Medicinal plants are considered as the very beneficent candidate to overcome the infectious related hazards e.g. Baba and Malik (2014) investigated the antioxidant and antibacterial activity of methanolic extract of *Gentianakurrooroyle* and they found that plant was very good.

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Dicliptera roxburghiana, belongs to the family Acanthaceae, is a perennial herb with 2-7 dm long stems. Leaves are green, flowers are arranged in axillary cymes having purple colour. (Wanger *et al*., 1999). Saturated fatty acids (C-15 to C-31) and flavonoids (apigenin, kaempferol luteolin and apigenin-7-O-glucoside) were isolated and identified from *Dicliptera roxburghiana* (Bahuguna *et al*., 1987). Ahmad et al (2013). demonstrated the antioxidant potential of *D. roxburghiana* using various in vitro antiradical test systems and they found that plant was very good candidate of antioxidant aptitude. As there is no published data for the antimicrobial and cytotoxic potential of *D. roxburghiana,* so this study was designed to investigate the antimicrobial activity along with cytotoxicity potential of plant.

MATERIALS AND METHODS

Plant extraction and fractionation Plant material

Dicliptera roxburghiana, collected from Quaid-i-Azam University, was identified by Prof. Dr. Rizwana Aleem Qureshi, Department of plant sciences, Quaid-i-Azam University, Islamabad and a voucher specimen (accession # 125521) was submitted in the Herbarium of Pakistan at Quaid-i-Azam University, Islamabad. Shade dried leaves were pulverized to form dry powder (2kg) which was extracted with 4.0 L methanol and filtered, the re-extraction of the residue was repeated twice. Filtrate was dried under rotary vacuum evaporator at 40ºC to obtained concentrated dry extract. To get further fractions 4.0g

methanol extract (DRME) was suspended in 200 mldistilled water followed by successive partitioning with n-Hexane, chloroform, ethyl acetate and n-butanol as shown in the fig. 1. Each fraction was dried and used for further analysis.

Antibacterial assay

Agar well diffusion method was used to evaluate the antibacterial activity (Bibi *et al.,* 2011). Stock of 15 mg/ml DMSO was used to get further dilutions of 12, 10, 7, 5, 3 and 1mg/ml. Antibiotic Cefexime (2mg/ml) was taken as positive control while DMSO was taken as negative control.

Bacterial strains

Eight bacterial strains were used. Among them three were Gram positive Bacillus Subtilis (ATCC 6633), Staphylococcus aureus (ATCC 6538) and Micrococcus luteus (ATCC 10240) and three were Gram negative named *Escherichia coli* (ATCC 15224), *Salmonella typhi* (ATCC 19196) and *Klebsiella pneumoniae* (MTCC 618).

Procedure

20 g/L Nutrient agar medium was dissolved in distilled water (pH 7.0) was autoclaved. At temperature 45ºC, 10 ml of freshly grown inocula was added to the agar media. Afterward 75ml of media was poured into labeled Petri plates of diameter 14 cm and was allowed to solidify. 10 wells per plate were made with sterile cork borer (8mm) and were sealed with 15µl of liquid agar media. 100 µl of each test sample was poured into respective well. As triplicate plates were assayed, mean zone of inhibition (mm) and minimum inhibitory concentration (MIC) were noted after 24 hrs and calculated by following formula:

$$\%\ \text{growth inhibition} = \left(\frac{\text{Test sample - solvent control}}{\text{Positive control}} \right) \times 100$$

Antifungal Assay

Protocol of Duraipandiyan and Ignacimuthu (2009) was followed to evaluate the antifungal activity against six fungal strains named *Aspergillus flavus* (0064), *Aspergillus fumigatus* (66), *Aspergillus niger* (0198), *Fusarium solanii* (0300). Sebarose dextrose agar media (MERCK) was used to carry out this assay. Stock solution of 12 mg/ml was prepared in DMSO. Turbinafine and DMSO were used as positive and negative controls respectively.

Assay Procedure

4 ml autoclaved media (6.5g/100ml dis. Water; pH: 5.5) was poured in autoclaved test tubes marked 10 cm. Sample (67µl) was mixed with media and tubes were placed to form slant. Seven days old cultures were inoculated on these agar slants and tubes were placed at 28ºC for seven days. Linear growths of fungus in test tubes were noted and growth inhibition was calculated as:

$$\text{Fungal growth \% Inhibition} = \frac{100 - \text{Linear growth in test tube (mm)}}{\text{Linear growth in control(mm)}} \times 100$$

Brine shrimps cytotoxic assay

Protocol of Meyer-Albert *et al.* (1992) was used to perform this assay. Sample dilutions (10, 100 and 1000 µg/ml) were made from stock solution (10mg/ml). Commercial sea salt (28 g/L) in distilled water was stirred for 2 hours continuously and poured in a container with two compartments. Eggs were sprinkled in dark compartment of container and were covered with aluminum foil and container was placed under florescent lamp for 24 hours. Hatched larvae were moved toward the lightened side of container and were ready to use.

Assay procedure

0.5ml of each solution was poured into drum vials and solvent was evaporated. 2ml of saline was added to these residues. 10 shrimps were added to each via land incubated at 28C. After 24 hour incubation living larvae were counted by 3x magnifying glass and calculation were obtained by using Abbot's formula;

% Death= (Sample -control)/control× 100.

STATISTICAL ANALYSIS

Prism graph pad software version V was used to calculate the LD_{50} of cytotoxicity assay and % inhibition values of antimicrobial assays with mean and standard deviations.

RESULTS

The basic aim of the study was to assess the antimicrobial and cytotoxic behavior of the plant. For this purpose six bacterial strains were tested to evaluate the antibacterial activity of the plant derived fractions. Table 1 describes the minimum inhibitory concentration of different extracted fractions against tested strains. Fig. 2 describes the zone of inhibitions displayed by various extracted fractions. DRME and DRHF induced effective inhibitions against all gram positive and gram negative bacteria with MIC value 3mg/ml against all bacterial strains where as DRCF was inhibiting *B. sub*, *M. leu*, and *S. typhy* at MIC 3mg/ml whereas for *S. aur, E. coli* and *K. pneumo* it was 5mg/ml. DREE was also effective against *S. aur, M. leu* and *E. coli* at MIC of 3mg/ml but in case of *B. sub* it was 5mg/ml and for *S. typhy* and *K. pneumo* it was 7mg/ml.

MIC value of DRBE was 3 mg/ml against *S. aur* and *M. leu* but against *B. sub, E. coli* and *S. typhy*it was 5mg/ml and against *K. pneumo* DRBF was displaying MIC of 7mg/ml. *D. roxburghiana* exhibited a very good antifungal activity which is described in table 2.

DRME and DRHF showed more than 70% inhibition against all fungal strains tested hence proven as strong antifungal agents. DRME inhibited the *A. niger, A. flavus,*

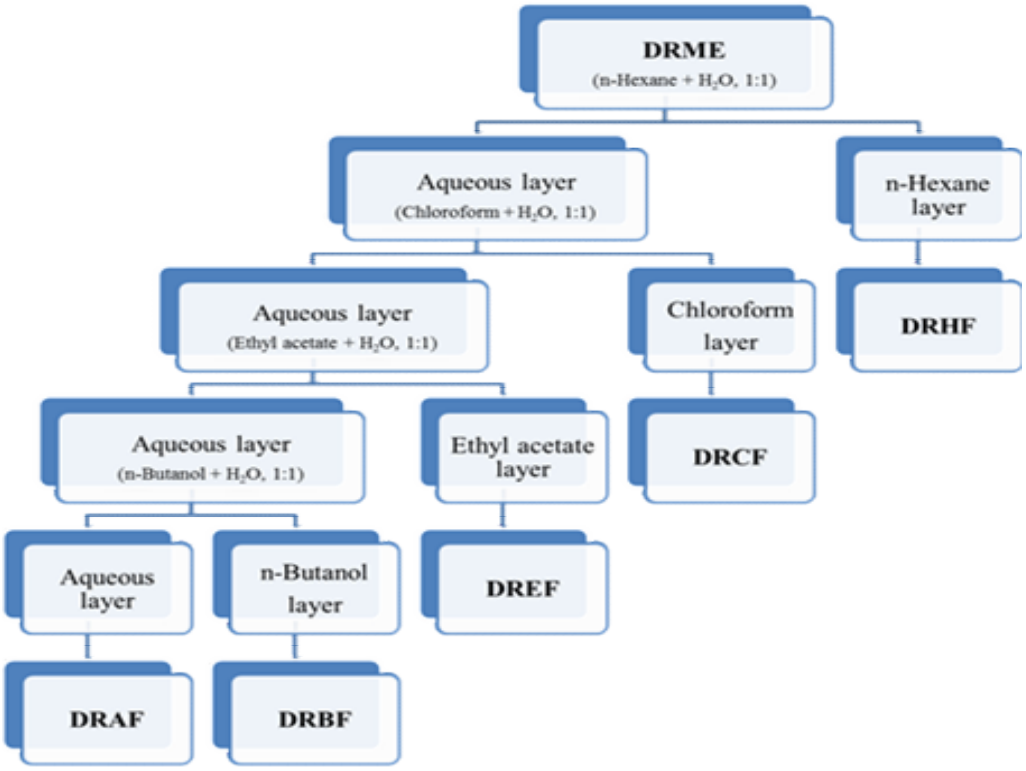

Fig. 1: Fractionation scheme of *D. roxburghiana*

Table 1: MIC and ZI values of extracted fractions of *D. roxburghiana* against different bacterial strains

Extract	Minimum inhibitory concentration (mg/ml)					
	S. aureus	*B. subtilis*	*M. luteus*	*E. coli*	*S. typhy*	*K. pneumonia*
DRME	3	3	3	3	3	3
DRHF	3	3	3	3	3	3
DRCF	5	3	3	5	3	5
DREF	3	5	3	3	7	7
DRBF	3	5	3	5	5	7
DRAF	7	5	5	7	10	7

MIC= Minimum inhibitory concentration (mg/ml).

A. fumigatus and *F. solani* with inhibition percentage of 81.13±2.33%, 72.50±1.13%, 79.46±1.91% and 77.48±1.37% respectively.

DRHF displayed growth inhibition at 78.63±1.94%, 75.41±0.87%, 81.62±1.41% and 78.54±1.27% for *A. niger, A. flavus, A. fumigatus* and *F. solani* respectively as shown in table 2.

DRCF, DREF and DRBF were behaving moderately for the fungal growth restriction. Minimum and maximum inhibition displayed by DRCF ranged from 51.86±1.25% *A. fumigatus* respectively.

Regarding DRBF inhibition was observed in the range of 63.26±1.38% as minimum value to 77.23±1.20% as maximum value for *A. niger* and *F.solani* respectively as described in table 2.

to 65.18±0.95% for *F. solani* and *A. flavus* respectively and DREF showed inhibition range from 32.91±1.29% (minimum) to 53.63±1.41% (maximum) for*F. solani* and DRAF was also exhibiting appreciable fungal growth inhibition ranging from 47.3±2.28% for *A. niger* to 60.1±5.12% for *F. solani*.

Cytotoxic activity of the plant was also determined by brine shrimps assay and a good % inhibition was exhibited by different plant fractions. DRME, DRHF, DRAF and DRBF showed strong cytotoxic % inhibition with LD_{50} values of 215, 199, 392 and 267µg/ml respectively.

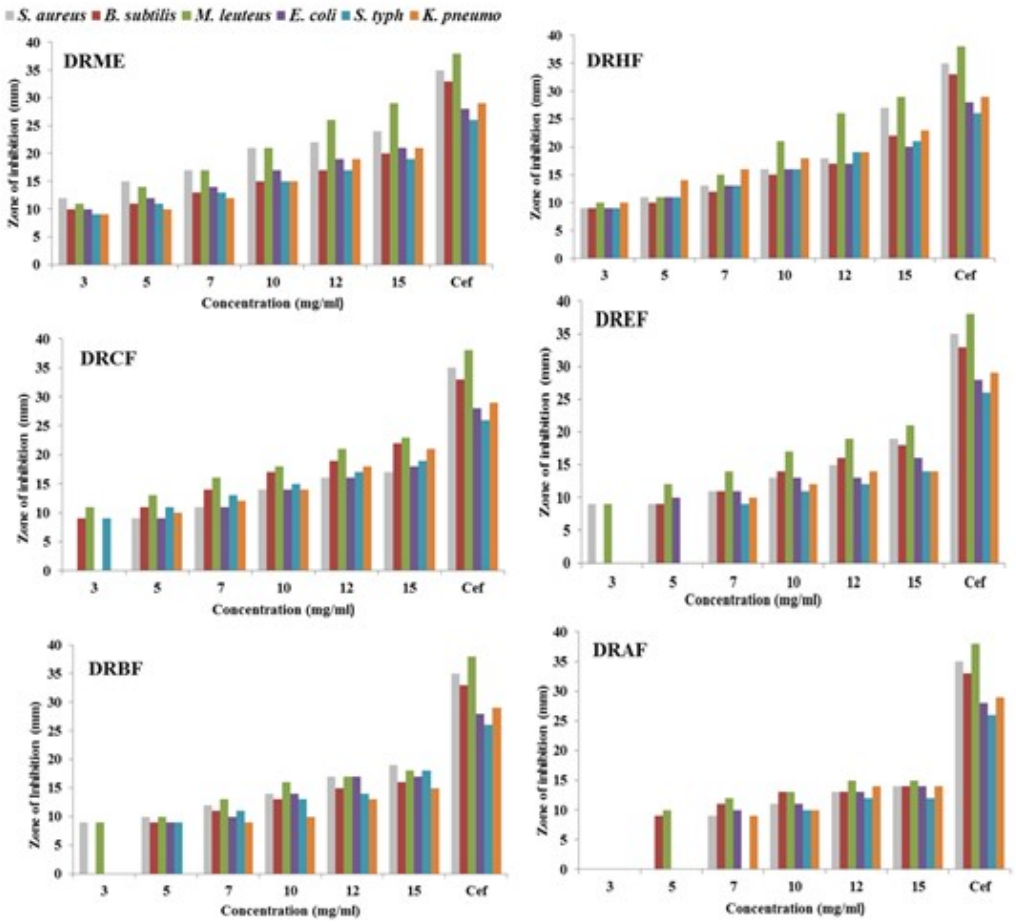

Fig. 2: Antibacterial activity of different extracted fractions showing zone of inhibitions (mm) at various concentrations (3-15 mg/ml). DRME: *D. roxburghiana* methanol extract; DRHF: *D. roxburghiana* n-Hexane fraction; DRCF: *D. roxburghiana* chloroform fraction; DREF: *D. roxburghiana* ethyl acetate fraction; DRBF: *D. roxburghiana* n-butanol fraction; DRAF: *D. roxburghiana* aqueous fraction.

Table 2: % inhibition of different extracts against fungal strains

Extract	Percentage inhibition (%)			
	A. niger	*A. flavus*	*A. fumigatus*	*F. solani*
DRME	81.13±2.33	72.50±1.13	79.46±1.91	77.48±1.37
DRHF	78.63±1.94	75.41±0.87	81.62±1.41	78.54±1.27
DRCF	60.16±2.34	65.18±0.95	58.74±1.21	51.86±1.25
DREF	48.93±1.70	37.46±1.89	53.63±1.41	32.91±1.29
DRBF	63.26±1.38	66.96±1.45	73.68±1.67	77.23±1.20
DRAF	47.3±2.28	56.4±2.21	54.6±3.02	60.1±5.12
Terb	86.56±0.85	88.26±1.32	89.03±1.35	82.83±1.78

A. Niger Aspergillus niger, A. flavus Aspergillus flavus, A. fumigat Aspergillus fumigatus F. solanii Fusarium solani, Terb Terbinafine *Data represents the mean value of triplicates.

DISCUSSION

This scenario showed a strong antimicrobial potential of plant extracts and fractions as described in results. Regarding antibacterial activity, overall DRME, DRCF, DREF and DRHF induced a significant inhibition against all tested strains which may be due to their phytoconstituents such as phenolics, tannins, saponins and flavonoids. A number of studies indicated that antimicrobial manifestations of plant extracts are due to their phenolics and flavonoid contents (Baba and Malik, 2014; Baydar *et al*., 2006; Mohanta *et al*., 2007). Another investigation was reported by Kabuki *et al*. (2000) that catechins were more efficient against gram positive

Table 3: Cytotoxic activity of *D. roxburghiana*

% Inhibition of extracts against Brine Shrimps				
Extract	10 (µg/ml)	100 (µg/ml)	1000 (µg/ml)	LD_{50}
DRME	32.8±0.99	55.8±0.08	68.1±0.97	215±0.16
DRHF	26.2±0.26	51.6±1.77	65.7±1.11	199±0.21
DREF	21.8±1.76	33.9±1.87	42.3±0.92	741±0.96
DRCF	15.7±1.91	29.2±2.76	48.7±1.66	673±1.09
DRBF	19.2±1.07	36.8±1.05	73.9±0.03	267±0.55
DRAF	36.8±0.82	58.2±0.04	77.8±0.09	392.8±0.26

Data represents the mean value of triplicates.

bacteria than gram negative bacteria. This efficiency was totally dependent on the structural differences between gram positive and negative bacteria; presence of outer membrane in gram negative bacteria interferes with the diffusion of some hydrophobic compounds across it. Tannins may easily diffuse through this outer membrane and have the ability to disrupt the proton motive force (PMF), active transport, electron flow and coagulation of cell contents (Burt, 2004). Therefore these structural modifications play a vital role their mode of susceptibility.

The plant extracts were active against fungal strains. A number of reports determined the antimicrobial activity of plants (Stepanovic *et al*., 2003; Bylka *et al*., 2004), alkaloids (Klausmeyer *et al*., 2004), flavonoids (Sohn *et al*., 2004) and diterpenes (EI-Seedi et *al*., 2002). Essential oils are also important to inhibit the fungal growth (Villa *et al*., 2002).

Cytotoxic analysis of extract and all fractions determined that there was no correlation between antibacterial activity and cytotoxic activity that led to the confirmation that there is another specific inhibitory mechanism involve in the antibacterial activity. Our results are in accordance with that of Al- Fatimi *et al*. (2007).

CONCLUSION

This research design concluded that plant possess strong antimicrobial potential that can play important role for the treatment of microbial infections. Compounds responsible for this activity can be used pharmaceutically in the synthesis of antibiotics to overcome the emerging resistance. Furthermore these natural antimicrobial entities would be more safer, cheaper and reliable than their synthetic counterparts.

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REFERENCES

Ahmad B, Khan MR, Shah N.A., Khan, R.A., 2013. *In vitro* antioxidant potential of *Dicliptera roxburghiana*. *BMC Complement Altern. Med.,* **13**: 140.

Assob JC, Kamga HLF, Nsagha DS, Njunda AL, Nde PF, Asongalem EA, Njouendou AJ, Sandjon B and Penlap VB (2011). Antimicrobial and toxicological activities of five medicinal plant species from Cameroon Traditional Medicine. *BMC Complement Altern Med.,* **11**: 70.

Al-Fatimi M, Wurster M, Schroder G and Lindequist U (2007). Antioxidant, antimicrobial and cytotoxic activities of selected medicinal plants from Yemen. *J Ethnopharmacol.,* **111**(3): 657-666.

Alviano DS and Alviano CS (2009). Plant extracts: Search for new alternatives to treat microbial diseases. *Curr. Pharm. Biotechnol.,* **10**(1): 106-121.

Baba SA and Malik SA (2014). Evaluation of antioxidant and antibacterial activity of methanolic extracts of *Gentiana kurroo* Royle. *Saudi J. Biol. Sci.*, **21**(5): 493-498

Bahuguna RP, Jangwan JS, Kaiya T and Sakakibara J (1987). Flavonoids and Fatty Acids of *Dicliptera roxburghiana*. *Pharm Biol.,* **25**(3): 177-178.

Baydar NG, Sagdic O, Ozkan G and Cetin S (2006). Determination of antibacterial effects and total phenolic contents of grape (*Vitis vinifera* L.) seed extracts. *Int. J. Food Sci. Technol.,* **41**(7): 799-804.

Bibi Y, Nisa S, Chaudhary FM and Zia M (2011). Antibacterial activity of some selected medicinal plants of Pakistan. *BMC Complement. Altern. Med.,* **11**: 52.

Burt S (2004). Essential oils: Their antibacterial properties and potential applications in foods. *Int. J. Food Microbiol.,* **94**(3): 223-253.

Bylka W, Szaufer-Hajdrych M, Matalawskan I and Goslinka O (2004). Antimicrobial activity of isocytisoside and extracts of *Aquilegia vulgaris* L. *Lett. Appl. Microbiol.,* **39**(1): 93-97.

Duraipandiyan V and Ignacimuthu S (2009). Antibacterial and antifungal activity of flindersine isolated from the traditional medicinal plant, *Toddalia asiatica* (L.) Lam. *J. Ethnopharmacol.,* **123**(3): 494-498.

El-Seedi, H.R., Sata, N., Torssell, K.B.,Nishiyama, S., 2002. New labdenediterpenes from *Eupatorium glutinosum*. *J. Nat. Pro.,* **65**(5): 728-729.

Gardam MA (2000). Is methicillin-resistant *Staphylococcus aureus* an emerging community pathogen? A review of the literature. *Can J. Infect Dis.,* **11**(4): 202-211.

Hemaiswarya S, Kruthiventi AK and Doble M (2008). Synergism between natural products and antibiotics against infectious diseases. *Phytomedicine,* **15**(8): 639-652.

Kabuki T, Nakajima H, Arai M, Ueda S, Kuwabara Y, Dosako S (2000). Characterization of novel antimicrobial compounds from mango (*Mangi feraindica* L.) kernel seeds. *Food Chem.,* **71**(1): 61-66.

Khan RA, Khan MR, Sahreen S and Bokhari J (2010). Antimicrobial and phytotoxic screening of various fractions of *Sonchusasper. Afr J. Biotechnol.,* **9**(25): 3883-3887.

Klausmeyer P, Chmurny GN, McCloud TG, Tucker KD and Shoemaker RH (2004). A novel antimicrobial indolizinium alkaloid from *Anibapanurensis*. *J. Nat. Pro.,* **67**(10): 1732-1735.

Meyer-Alber A, Hartmann H, Sumpel F, Creutzfeldt W, (1992). Mechanism of insulin resistance in CCl_4-induced cirrhosis of rats. Gastroenterology, **102**(1): 223-229.

Mohanta TK, Patra JK, Rath SK, Pal DK and Thatoi HN (2007). Evaluation of antimicrobial activity and phytochemical screening of oils and nuts of *Semicarpus anacardium* L. f. *Sci. Res. Essay,* **2**(11): 486-490.

Sohn HY, Son KH, Kwon CS, Kwon GS and Kang SS (2004). Antimicrobial and cytotoxic activity of 18 prenylated flavonoids isolated from medicinal plants: *Morus alba* L., *Morus mongolica* Schneider, *Broussnetia papyrifera* (L.) Vent *Sophora flavescens* Ait and *Echinosophora koreensis* Nakai. *Phytomedicine*, **11**(7-8): 666-672.

Stepanovic S, Antic N, Dakic I and Svabic-vlahovic M (2003). *In vitro* antimicrobial activity of propilis and antimicrobial drugs. *Microbiol. Res.,* **158**(4): 353-357.

Vila R, Mundina M, Tomi F, Furlan R, Zacchino S, Casanova J and Canigueral S (2002). Composition and antifungal activity of the essential oil of Solidagochilensis. *Planta Medica,* **68**(2): 164-167.

Wendakoon C, Calderon P and Gagnon D (2011). Evaluation of selected medicinal plants extracted in different ethanol concentrations for antibacterial activity against human pathogens. *JMAP.,* **1**(2): 60-68.

Wagner Warren LH, Derral R and Sohmer SH (1999). Manual of the flowering plants of Hawaii. Revised edition. Bernice P. Bishop Museum special publication. Honolulu, Hawaii.