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Biopotentials of secondary metabolites isolated from marine sponges

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Abstract

The secondary metabolites of three sponges collected as bycatch in the fishing nets were explored for biological potencies. The sponge *Dendrilla nigra* exhibited wider biological activity. It showed potent activity in antibacterial, brineshrimp cytotoxicity, larvicidal, antifouling and ichthyotoxic assays. One of the well-studied cytotoxic sponge *Axinella donnani* was least active in brineshrimp cytotoxicity assay. The secondary metabolites of *Clathria gorgonoides* were highly cytotoxic albeit it showed least activity in other bioassays. Based on the present findings, it could be inferred that the bioassay-guided fractionation and purification of *D. nigra* may come up with potent bioactive drugs.

Introduction

Marine Secondary Metabolites (MSMs) are organic compounds produced by microbes, sponges, seaweeds and other marine organisms (Attaway & Zaborsky, 1993). The host organism biosynthesizes these compounds as non-primary or secondary metabolites to protect themselves and to maintain homeostasis in their environment (Selvin, 2002). Some of these secondary metabolites offer avenues for developing potent drugs. The rapid growth in the chemistry of marine organisms over the last 15 years has led to the discovery of a large number of new structures, many of which have no precedence among structures of terrestrial origin and possess previously unknown pharmacological and toxicological properties.

Retrospective of research in this field indicated that although a number of diverse biologically active compounds have been isolated from marine organisms, the number of compounds taken-up for the field trial/clinical use is scanty. This may be due to the failure of successful collection of concerned source organism in bulk or which have same sort of secondary metabolites. Therefore exploration of chemical ecology of secondary metabolites synthesis and development of drugs from sponge-associated microorganisms are becoming a promising venture (Soniya, 2003). Although chemical synthesis of bioactive secondary metabolites have been developed, the availability of source organism in bulk is inevitable for systemic drug development. Successful development of drugs from the sea is completely relying on the availability of source organism or the organism having same secondary metabolites. Therefore the knowledge of habitat, areas of abundance, seasonality and eco-friendly bulk collection of the source organism are very important for the successful development of potent bioactive drugs. In this context, the present study was initiated to find out the biopotentials of marine sponges collected as bycatch in fishing nets.

Materials and methods

An eco-friendly bulk collection of sponges as bycatch in the fishing nets was carried out at Kanyakumari

coast (southeast coast of India). Three species of sponges were collected, identified and taken-up for isolation and bioactivity screening of secondary metabolites. During November to December and April to August of every year, the rough sea weather was prevailed in the Kanyakumari coast led dislodging of diverse sponges, gorgonids, soft corals and ascidians, which were caught in the fishing nets. These bycatches were segregated and collected freshly from the nets and washed in fresh seawater to remove dirt and symbionts and drained off the excess water on a blotting sheet. After recording their colour pattern for identification, the sponge pieces were separately preserved in methanol. For the isolation of secondary metabolites, they were minced in a tissue homogenizer (Omni, U.S.A.) and extracted with methanol and methanol-dichloromethane (1:1). The combined extract was filtered and concentrated in a rotary vacuum evaporator (Buchi) at room temperature.

Bioassays

Antibacterial activity of MSMs was studied using seven species of bacterial type cultures (MTCC) and four species of shrimp/fish pathogenic bacteria (obtained from Marine Biotechnology lab, Vizhinjam) as test organisms. The modified cylinder plate double layer method was used for the screening of potent antibacterial MSMs and minimum inhibitory concentration (MIC) values. In this method, the base layer was prepared with 10 ml of 1.5% agar. Six numbers of sterile porcelain beads of 7 mm diameter was placed on the base layer. The overlaid seed layer was prepared by pouring 15 ml of hot nutrient agar containing 0.2 ml of prepared inoculum (USP, 1995; The Himedia manual, 1998). After the seed layer solidified, the porcelain beads were removed carefully with a sterile forceps. The consequent wells were filled with the appropriate test compound and control. After 18 h of incubation at 37±2 °C, the area of inhibition zone was measured. A parallel experiment was conducted at 20±2 °C to find out the influence of temperature on the antibacterial potential of MSMs.

Brineshrimp cytotoxicity of MSMs was evaluated after Hong et al. (1998). Based on the percent mortality, the LC50 values of the test compound were determined by probit analysis (Wardlaw, 1985). The susceptibility or resistance of the mosquito larvae (*Culex* sp.) to the selected concentration of the extracts (larvicidal effect) was studied by adopting standard bioassay protocol (WHO, 1981). Fingerlings (1.5–2.0 cm) of marine acclimated *Oreochromis mossambicus* were used for evaluating the ichthyotoxic potential. Immediate reflex changes and mortality of fingerlings treated with various concentrations of MSMs were observed continuously for 6 h and at 1 h interval for the next 12 h. The acute toxicological reflexes were observed after Indap & Pathare (1998). The newly developed 'foot adherence bioassay' was used for the evaluation of antifouling activity of MSMs (Selvin & Lipton, 2002).

Results and discussion

Collection of the source organisms

In the present study, the Kanyakumari coast was found to be an excellent area for the collection of diverse marine sponges as bycatch in fishing nets. The landing status of sponges in this coast is more or less same, during the seasons (Lipton et al., 2003). However, the trend of frequency and quantity of landings varied according to the fishing area and nets used. The major landing species were *Dendrilla nigra, Clathria gorgonoides* and *Axinella donnani.* All these bycatch collections contained potent biologically active secondary metabolites. The recovery of extract was dependent on colour pattern and softness of the sponge body. The yield was very high in the case of *A. donnani* (8 g/kg) followed by *D. nigra* (6.0 g/kg) and *C. gorgonoides* (3.8 g/kg). Literature indicated that utilization of such bycatches for bioactivity screening and or chemical elucidation was scanty. However, Ovenden & Capon (1999) utilized a *Sigmosceptrella* sp. obtained from trawling operation in the Great Australian Bight for the chemical elucidation and bioactivity screening. A specimen of *Dendrilla cactos* collected during trawling operations in Bass strait, Australia had yielded two new alkaloids, lamellarino lamellarin-p, which was previously reported from tunicates and molluscs (Urban et al., 1994).

Antibacterial activity

The secondary metabolites of sponges exhibited significant bactericidal activity. The sponge *D. nigra* exhibited broad-spectrum antibacterial activity and it inhibited the growth of all the tested bacteria (Table 1). However, *A. donnani* was a narrow spectrum antibacterial agent as it inhibited the growth of all the gram positive bacteria while it showed least activity to the extent of 25.0% against gram negative bacteria.

Table 1. Antibiogram of MSMs

Source organism of MSMs	% antibacterial activity					
	Total (%)		Gram positive (%)		Gram negative (%)	
	37 °C	20 °C	37 °C	20 °C	37 °C	20 °C
Dendrilla nigra	100	100	100	100	100	100
Axinella donnani	45.45	45.45	100	100	25	25
Clathria gornonoides	27.27	18.18	66.66	66.66	12.5	0

Most of the available reports on antibacterial property of sponges revealed their activity on gram positive bacteria. Samples of 28 demosponges collected along French coast indicated high antibacterial activity against gram positive bacteria (77%) than gram negative bacteria (53%) (Amade et al., 1987). As *C. gorgonoides* contained least active secondary metabolite, it inhibited only 27.27% of the tested bacteria. The activity was very much reduced against gram-negative bacteria at 37 °C while it exhibited no activity at 20 °C.

Based on the activity range, the inhibitory potential was graded as highly active (>100 mm^2), nearly active (60–100 mm^2), moderately active (30 to <60 mm^2) less active (1 to <30 mm^2) and resistant (<1 mm^2). The bactericidal potential of *D. nigra* was very high against *Micrococcus luteus* to the extent of 132.66 mm^2 and 283.3 mm^2 at 37 and 20 °C respectively (Fig. 1). The high activity range was extended against one of the shrimp pathogen *Vibrio fischeri* with 104.62 mm^2 at 20 °C whereas the activity was very low at 37 °C. The antibacterial potential of *D. nigra* was also evidenced in 'in captivity' control of bacterial diseases of shrimp (Selvin, 2002). The nearly active range was observed against *Bacillus cereus* (78.8 mm^2) at 20 °C and *Pseudomonas aeruginosa* (63.58 mm^2) at both temperatures. Five bacterial strains were nearly sensitive to *D. nigra* at different temperatures. The activity range of *B. cereaus* and *Escherichia coli* accounted for 50.24 and 38.46 mm^2 zone, respectively, at 37 °C. One of the common shrimp pathogen *V. alginolyticus* (QS7) was moderately sensitive at 20 °C whereas luminescent *V. harveyi* (RJM5) was less sensitive in both temperatures. In the case of *B. subtilis*, it was moderately sensitive at both temperatures. The inhibitory potential was least against *A. hydrophila* and clown fish isolates (CF1 and CF2) at both temperatures.

In the case of *A. donnani*, peak bactericidal activity was noted against *A. hydrophilla* at 20 °C to the extent of 132.66 mm^2 (Fig. 2). However, at 37 °C, it was resistant. The antibacterial activity of *A. donnani* indicated its narrow spectrum as it inhibited the growth of all the three gram positive strains. The nearly active bactericidal range was observed against *B. cereaus* (63.58 mm^2) and *P. aeruginosa* (78.5 mm^2) at 20 °C. The activity range was reduced to moderate against *B. subtilis* at both temperatures, and *M. luteus* at 20 °C, however it was less active at 37 °C. The bacterial strains such as *E. coli*, *V. fischeri*, shrimp isolates and fish isolates produced resistant colonies against *A. donnani* at both the temperatures except CF1, which showed very least sensitivity (3.14 mm^2) at 37 °C.

The secondary metabolite of *C. gorgonoides* was one of the least active antibacterial agents, which inhibited the growth of 27.27 and 18.18% of total bacterial species tested at 37 °C and 20 °C, respectively (Table 1). The activity spectrum was narrowed towards gram negative bacteria, which inhibited the growth of 66.66%, while it was not active against gram positive bacteria at 20 °C. It showed moderate activity to the extent of 63.58 mm^2 inhibition area at 20 °C, with least activity at 37 °C (Fig. 3). It was moderately bacteriostatic against *B. cereus* at both temperatures and *P. aeruginosa* at 37 °C. One gram positive and 7 gram negative species were totally resistant against *C. gorgonoides* extract.

The present findings indicated that temperature had significant influence on the antibacterial potential of MSMs. In certain cases, the activity was comparatively high in 20 °C. It may be due to the slow growth rate of bacteria at low temperatures. The MIC value of highly active MSMs indicated that *D. nigra* was a potent antibacterial compound (MIC = 9.0 mg/ml) followed by *A. donnani* (MIC = 12.0 mg/ml) and *C. gorgonoides* (14.0 mg/ml). Literature indicated that sponges contained potent antibacterial secondary metabolites. The dichloromethane-methanol (1:1) extract of the sponge *Phycopsis* sp. collected from the Tuticorin coast of India, exhibited antibacterial activity (Venkateswarlu & Biabani, 1995). The bromopyrrole alkaloids found in *Agelas dispar* showed moderate antibiotic activity against gram positive bacteria such as *B. Subtilis* and *S. aureaus* (Caiferi et al., 1998). The latrunculid marine sponges *Latrunculia* sp. and *Negombata* sp. were contained potent antibacterial discorhabdin R, which was chemically characterized as pyrrolaiminoquinone (Ford & Capon, 2000). Extracts made from *Sigmosceptrella* sp. collected as bycatch during trawling operation in the Great Australian Bight was inhibitory against *M. luteus, Serratia marcescens* and *Staphylococcus aureus* (Ovenden & Capon, 1999). Based on the present findings, it could be inferred that the bycatch collections would form an

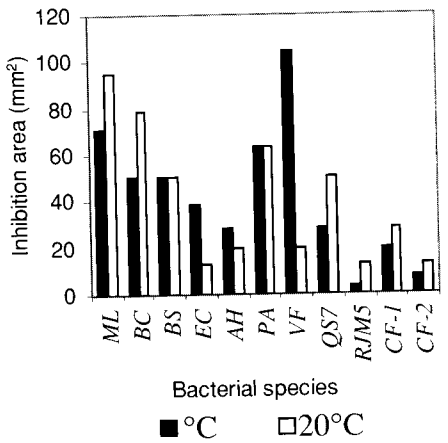


Figure 1. Antibacterial activity of *Dendrilla nigra*.

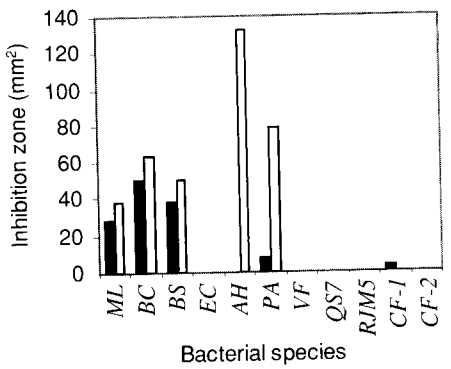
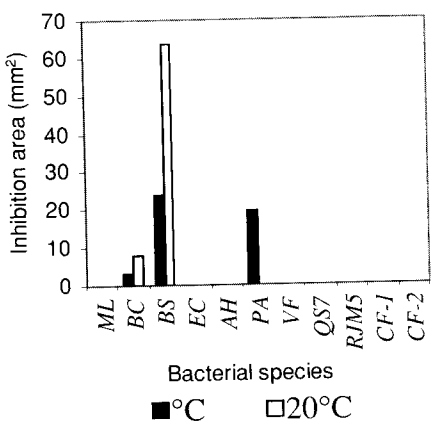


Figure 2. Antibacterial activity of *Axinella donnani*.



ML – Micrococcus luteus
BC – Bacillus cereus
BS – Bacillus subtilis
EC – Escherichia coli
AH – Aeromonas hydrophila
PA – Pseudomonas aeruginosa
VF – Vibrio fischeri
QS7 – Vibrio alginolyticus
RJM5 – Vibrio harveyi
CF-1 – Aeromonas sp.
CF-2 - Aeromonas sp.

Figure 3. Antibacterial activity of *Clathria gorgonoides*.

additional resource for developing potent antibacterial agents.

Brineshrimp cytotoxicity

Results of brineshrimp cytotoxicity bioassay are presented in Table 2. The secondary metabolites of the sponge *C. gorgonoides* exhibited high toxicity against *Artemia* nauplii. One of the well-studied cytotoxic sponge *A. donnani* reported with vast potential of anti-tumour activity (Bai et al., 1991; Rudi et al., 1997) was found to be least active in the present study. Probit analysis indicated that it was least toxic and it produced 50% mortality at 5.5% level. This lesser toxicity may be due to the geographical location or habitat of the sponges. The LC50 values of *C. gorgonoides* and *D. nigra* were accounted for 0.20% and 0.28%, respectively. Temperature had significantly influenced the toxicity of MSMs. The toxicity profile of MSMs considerably decreased at 20±2 °C (Table 2). At this temperature, the toxicity of *D. nigra* was reduced to no mortality at 0.4% level while the same concentration resulted in 90% mortality at 37±2 °C. Similarly *A. donnani* showed 80% mortality at 10% level and it produced 80% mortality at 37±2 °C. The same trend was noticed for *C. gorgonoides* also.

Methanol soluble extract of the Korean sponge *Petrosia* sp. showed significant activity in the brineshrimp cytotoxicity assay (LD50 = 30 μg/ml). Guided by this assay, further fractionation and purification pro-

Table 2. Brine shrimp cytotoxicity profile of MSMs at 30 and 20 °C

MSMs	Concentration (%)	Mortality (%) 30 ± 2 °C	20 ± 2 °C
D. nigra	0.2	20.0 ± 4.14	0
	0.4	90.0 ± 3.94	0
	0.6	100 ± 0.0	10.0 ± 1.26
A. donnani	2	10.0 ± 1.26	0
	4	30.0 ± 3.16	10.2 ± 2.5
	6	50.0 ± 4.93	21.2 ± 2.5
	10	80.0 ± 5.0	40.0 ± 5.15
C. gorgonoides	0.1	23.8 ± 6.25	0
	0.2	60.4 ± 6.46	0
	0.4	79.2 ± 8.12	20.0 ± 1.78
	0.6	100 ± 0.0	80.2 ± 4.74

Mean ±SD. $n = 10$ experiments.

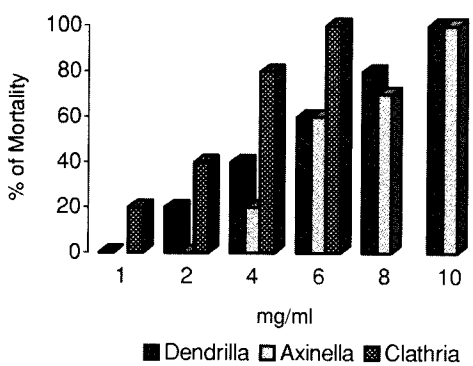


Figure 4. Larvicidal activity of MSMs.

cedures gave potent cytotoxic polyacetylenes (Kim et al., 1999). The Philippine marine sponge, *Plakinastrella* sp. yielded peroxide containing metabolites and the crude methanolic extract were toxic to brineshrimp (Quershi et al., 1998). Aglestatin A isolated from *Cymbastela* sp. (Axinellida) was highly toxic to brineshrimp (Hong et al., 1998). The crude extracts of marine sponges, *Pachastrella* sp. and *Jaspis* sp. collected from the south sea of Korea, exhibited significant brineshrimp cytotoxicity. Guided by this bioassay, fractionation and purification gave Pectenotoxin II and Psammaplin A, which were cytotoxic to human cancer cell lines (Jung et al., 1995). Therefore, bioassay guided purification of *C. gorgonoides* and *D. nigra* may give potent cytotoxic drugs.

Larvicidal effect

Larvicidal potentials of MSMs, based on the mortality of second instar larvae are depicted in Figure 4. The

Table 3. Ichthyotoxicity profile of MSMs to *Oreochromis mossambicus* fingerlings

Species	Concentration (%)	Mortality (%)	Time of death (h)
D. nigra	4	100	1
	2	60.6 ± 5.6	3
	1	10.0 ± 3.34	6
C. gorgonoides	4	100	2
	2	70.2 ± 4.26	3
	1	39.8 ± 4.0	6
A. donnani	4	100	50 Sec
	2	100	2
	1	40.8 ± 3.96	3

Mean ± SD. $n = 10$ experiments.

secondary metabolite of *C. gorgonoides* was found to be highly lethal to mosquito larvae. Larvicidal potential of *D. nigra* and *A. donnani* were more or less same and they produced 100% mortality at 10% level. Literature on the efficacy of sponge secondary metabolites on mosquito larvae was scanty. Methylicosadienoic acids isolated from the Caribbean sponge *Cymbastela* sp. (*Axinellida*) were contained larvicidal potential against beet army worm, *Spodoptera exigua* and corn rootworm *Diabrotica undecimpunctata* (Hong et al., 1998).

Ichthyotoxicity

Secondary metabolites produced by marine invertebrates were generally considered to play a role in the survival of host organism. They may be toxic or noxious and prevent predation, infection, and fouling or otherwise mediate ecological phenomena. This mechanism has been proved in several *in vitro* assays. Ichthyotoxic potential is considered as one of the mechanisms, which may indicate the feeding deterrent property of sponges to prevent predation. Although *C. gorgonoides* and *A. donnani* produced the same level of toxicity, *A. donnani* was considered as highly toxic and mortality was observed within 50 s at 4% level while it induced mortality after 2 h, in the case of *C. gorgonoides* (Table 3). At 1% level, *D. nigra* exhibited 10% mortality at 6 h of post-exposure. The *Spongia* sp. reported to produce two classes of terpenoid toxin, the cytotoxic sponginolides and ichthyotoxic kurospongin (Jayatilake & Baker, 1996). Crambines from *Crambe crambe*, were found to be one of the most toxic and widespread species in rocky sublittoral habitats of Mediterranean sea due to its antipredation

Table 4. General behavioural changes observed in *Oreochromis mossambicus* exposed to ichthyotoxic MSMs

Stages	Behavioural changes
State I:	a) Increased in ventilatory frequency
Initial signs	b) Erratic /rapid movements
Stage II:	a) Inclined towards one side
Secondary signs	b) Loss of swimming activity
State III:	a) Rapid surface respiration
Advanced signs	b) Inclined to bottom
	c) Start of sporadic uncontrollable swimming with non directional bursts

Table 5. Antifouling activity of secondary metabolites isolated from sponges

Species	Concentration of extract (mg/ml)	Fouling rate (%)	Regaining rate (%)
Dendrilla	1.92	65 ± 5.4	100
	2.84	30 ± 7.0	100
	5.68	20 ± 8.9	100
	11.36	0	100
	19.2	0	80
Axinella	12.5	0	20
	25.0	0	0
Clathria	46.1	10 ± 7.0	100

Mean ± SD. $n = 10$ experiments.

(ichthyotoxic) activity (Berlinck et al., 1992; Becerro et al., 1997).

All the MSMs exhibited more or less same sort of behavioural changes (acute toxicological reflexes) (Table 4). Initially the fishes exhibited erratic movements and then inclined towards one side. Later, they rapidly went for surface respiration followed by settling at bottom or rapid swimming activity with non-directional bursts, which culminated in dwelling at bottom and mortality (Indap & Pathare, 1998).

Antifouling activity

The relative potency of antifouling secondary metabolites isolated from sponges were graded as more potent to weak as *D. nigra* > *A. donnai* > *C. gorgonoides* in the decreasing order. *D. nigra* was more effective and safe. It completely prevented the foot adherence of common rock fouler *Patella vulgata* at 11.36 mg/ml (Table 5). As no earlier reports were available regarding isolation of antifouling active principles from *D. nigra,* this study forms the first report of antifouling compounds in the species. Albeit *A. donnani* prevented fouling of *P. vulgata* at 12.5 mg/ml, only 20% of exposed *P. vulgata* were survived in fresh seawater. According to earlier reports, the high toxicity in *A. donnani* could be attributed to the presence of cytotoxins (Pettit et al., 1991; Rudi et al., 1995). In the case of *C. gorgonoides*, it was least active and prevented only 10% of foot adherence even at the higher concentration of 46.1 mg/ml. Based on the present findings, it could be inferred that the extracts from the sponge *D. nigra* was new, having potent antifouling activity, which can be further used for bioassay guided purification.

If the overall bioactivity profile is considered for discussion, it could be inferred that the extract of a single species showed a wide range of bioactivity. For example, *D. nigra* exhibited potent antibacterial, brineshrimp cytotoxicity, larvicidal, antifouling and antipredation ichthyotoxic) activities. The actual mechanism of such a broad-spectrum of bioactivity exhibited by a single species was not known. As crude extracts only were used, they may contain more than one compound or active principles. Earlier reports on the secondary metabolites such as Puupehenone and its related metabolites isolated from *Hyrtios* sp. showed similar potent antibacterial, antiviral, antifungal, cytotoxic and immunomodulatory activities (Pettit et al., 1998 and references therein). Molokaiamine metabolite from *Aplysinella* sp. showed antiviral, antifouling and cytotoxic activities (Fu & Schmitz, 1999 and references therein). Manzamine-type alkaloids isolated from the Philippine marine sponge *Xestospongia ashmorica* showed insecticidal, antibacterial and cytotoxicity activities (Edrada, et al., 1996). Cacospongionolide, B a new sesterpene, isolated from *Fasciospongia covernosa* showed antimicrobial activity, brineshrimp cytotoxicity and ichythyotoxicity (de-Rosa et al., 1995). Contrary to the earlier reports of *Axinella, A. donnani* showed lower activity in the antimicrobial and brineshrimp cytotoxicity assays (Uriz et al., 1992; Barrow & Capon, 1993; Bandaranayake, 1994; Rudi et al., 1997).

In accordance with the present study, crude extract of *Clathria* sp. showed significant feeding deterrent, cytotoxic and haemolytic activities (Kamiya et al., 1990; Wright et al., 1997). The feeding deterrent activity was reported as a chemical defense in Antartic sponge *Dendrilla membrosa* (Baker et al., 1995). The

wider biosynthetic capabilities of sponges are associated with their symbiotic microorganisms. Ivanova et al., (1993, 1994) reported the symbiotic bacterial association with *Dendrilla* sp. The predominant species belong to vibrionaceae (35%) and *Bacillus* (35%). These bacterial strains were capable of synthezing antibiotics (30% of total strain) and cytotoxin (20%). Two strains of *Bacillus pumilus* produced amicoumacine antibiotics (Ivanova et al., 1994). Therefore the highest bioactivity potential of *D. nigra* observed in the present study could be linked to the symbiotic bacterial association. Research is being going-on on the aspects of mechanism of secondary metabolites synthesis and development of drugs from sponge associated bacteria. Based on the present findings, it could be envisaged that the bioassay guided purification and fractionation of *D. nigra* may give-forth potent antimicrobial, anticancer and insecticidal agents.

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