ISSN 0974 – 5211

Journal of Natural Products

Volume 5 (2012)

www.JournalofNaturalProducts.Com

Research Paper

Antimicrobial activity of naphthoquinones extracted from *Arnebia nobilis*

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(Received 15 March 2012; Revised 21 March-20 May 2012; Accepted 20 May 2012)

ABSTRACT

Arnebia nobilis Rech. f., a natural source of red dye has traditionally been used as a food colourant, in cosmetic formulations and pharmaceutical preparations. In this study, the extracted dye and separated components of *A. nobilis* have been studied for their antibacterial activity. The extracted dye and its major component, alkannin β, β-dimethylacrylate has also been evaluated as an antibacterial finish on various textile substrates viz. nylon, polyester, silk, wool, cotton and acrylic. The dye and its components showed excellent antimicrobial activity against both *S. aureus* and *E. coli*. Amongst the fabrics dyed with 5% dye, wool, silk and acrylic showed 100% activity against both the microbes. Polyester showed 100% activity against *S. aureus* and ~ 80% activity against *E. coli*. Nylon and cotton showed no antimicrobial activity. Durability of antimicrobial activity to laundering and to light is also discussed.

Keywords: *A. nobilis*; Alkannin β,β-dimethylacrylate; Dyeing; Antibacterial; Naphthoquinones.

INTRODUCTION

Textiles find immense applications in day to day life and there has been a growing need to develop finishes for textile materials that can offer improved protection to the users from microbes. The large surface area and the ability to retain moisture make textile fabrics more prone to bacterial growth. Hence, there is a pressing need to develop textiles that are resistant to microbes as the textile substrates find various medical applications apart from conventional apparel usage (Giridev, et al., 2009; Singh, et al., 2005).

Protection against microbes is obtained by using antibacterial finished textile products. Examples of agents that have been successfully used as antibacterial agents on textile substrates include silver (Singh, et al., 2008; Irwin, et al., 2010), 2, 4, 4'-Trichloro-2'-hydroxydiphenyl ether (Firehammer, 1987) and quaternary ammonium compounds (McBain, et al., 2004; Xiao, et al., 2008). However, over a period of time bacteria generally tend to develop resistance to almost all agents rendering them ineffective for extended use. Thus, it is necessary that the innovative sources of

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antibacterial agents are sought in order to battle the growing problem of antibacterial resistant bacteria.

Many of the natural dyes derived from plant sources are classified as medicinal and possess remarkable antimicrobial activity (Giridev, et al., 2009; Singh, et al., 2005). Plant dyes rich in naphthoquinones such as lawsone from henna, juglone from walnut and lapachol from alkannet are reported to exhibit antibacterial and antifungal activity (Singh, et al., 2005; Joshi, et al., 2009). One such natural source of naphthoquinones is *Arnebia nobilis* Rech.f. (Family-*Boraginaceae*), referred to as 'Ratanjot', is an herbaceous and perennial plant with a height of 20-90cm, 'Ratanjot' does not grow in India (Arora, et al., 2009); however, the roots of this plant are imported into India from Afghanistan. Arora and her co-workers (2009) found that 'Ratanjot' roots are available in Indian bazaars as purple brown pieces of roots and rootstocks, covered with several layers of thin scaly bark of the same colour.

As reported earlier (Arora, et al., 2012), the n-hexane extract (A) of *A. nobilis* contains five different colourants. Chromatographic and spectroscopic analysis established that all the colourants are derivatives of alkannin. Alkannin β, β-dimethylacrylate (5,8-dihydroxy-2-(1'-β,β-dimethylacryloxy-4'-methylpent-3'-enyl)-1,4- naphthoquinone) (B) was identified as the major component (25%) followed by alkannin acetate (2-(1'-acetoxy-4'-methylpent-3'-enyl)-5,8-dihydroxy-1,4-naphthoquinone) (C) and shikonin (5,8-dihydroxy-4'-methylpent-3'-enyl)-1,4-naphthoquinone) (D).

These naphthoquinones have a long established history of being used as an antiseptic, antipyretic and anthelmintic substance, in eye diseases, bronchitis, abdominal pain and as an anti-inflammatory agent (Khatoon, et al., 1994; Cheng, et al., 1995; Papageorgiou, et al., 2002; Indrayan, et al., 2004; Spyros, et al., 2005; Akkol, et al., 2009). Besides these properties, the root bark of *A. nobilis* is reported to have several other biological properties such as antimicrobial, antifungal, antiviral, antitumor, antioxidant, radical scavenging and antithrombotic activity (Khatoon, et al., 1993; Cheng, et al., 1995; Papageorgiou, et al., 2002; Chen, et al., 2002; Khatoon, et al., 2003; Spyros, et al., 2005; Akkol, et al., 2009). Although few studies have been reported in literature on the antimicrobial activity of the crude dye extract (Patel and Patel, 1966; Shukla, et al., 1969; Papageorgiou, et al., 1979; Shen, et al., 2002; Jain, et al., 2003; Naz, et al., 2006) however, detailed studies are limited. Moreover, not many studies have been reported on the antimicrobial activity of the components of this plant extract.

There is one application study in which the efficacy of shikonin extracted from *Lithospermum erythrorhizon* as an antibacterial agent on silk was investigated (Dhandapani and Sarkar, 2007).

In view of the above literature, it is clear that despite of the therapeutic properties of *Arnebia*, the roots of *A. nobilis* have not been exploited fully with reference to its components and bioactive potential. Therefore, in this study, an attempt has been made to study the antimicrobial property of the purified components along with the crude dye extract of *A. nobilis.* The results have been compared with the standard sample of shikonin (S) and the minimum inhibitory concentration (MIC) is established. As textiles show preferential absorption in a dyebath, the behavior of dye on textiles cannot always be predicted from its behavior in solution form. Dye-fibre interactions can also affect the antimicrobial properties of a dyed textile. Therefore, the crude and purified component, alkannin β, β-dimethylacrylate have been applied to various textile substrates viz. nylon, polyester, wool, silk, cotton,

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acrylic and their antimicrobial activity has been assessed. Durability of the antimicrobial activity to laundering and light exposure has also been studied.

MATERIALS AND METHODS

Plant material: Dried root material of *Arnebia nobilis* was procured from Nature and Nurture Healthcare Private Limited, Delhi, India. Roots of the plant were ground to coarse powder and extracted in a single cycle lasting 8.5 hours with n-hexane in soxhlet apparatus, at 45°C. A deep red viscous residue amounting to ~5% on the dry weight basis of roots was obtained after evaporation of solvent.

***Dye samples*:** The crude dye extract was subjected to column chromatography. Purified components B, C, D were collected using n-hexane: ethyl acetate (2%, 3%, 4% respectively). Reference substance shikonin (S) isolated from *Lithospermum erythrorhizon* was procured from Merck, (≥ 97% purity by TLC, batch number 517-89-5). 0.5% stock solution of all samples A, B, C, D and S was prepared in methanol for the testing of antimicrobial activity.

Dyed fabrics: Since enough dye sample was not available for C, D and S, therefore, dyeing studies were conducted only with A and B on polyester, nylon, wool, silk, cotton and acrylic. The substrates were dyed with 0.5% and 5% shade owf for 60 minutes at pH 4.5. The dyebath also contained 01g/L sodium lauryl sulphate (SLS) as dispersing agent and liquor-to-goods ratio was maintained at 30:1. Polyester was dyed at 100°C and 130°C. Cotton, nylon, wool, silk and acrylic were dyed at 80°C. The dyed samples were then cold rinsed and soaped with 0.5g/L Lissapol N at 50ºC for 20 minutes. The samples were then cold rinsed and dried.

***Test organisms and chemicals used for antimicrobial testing*:** *Staphylococcus aureus* (NCIMB-17) and *Escherichia coli* (NCIMB -1) used in the study were obtained from the culture collection of Department of Biochemical Engineering and Biotechnology, IIT Delhi, India. Luria broth (Hi-media) was used to maintain the liquid culture and agar- agar was used to maintain the solid culture on the sterile petridish. All other chemicals used were of analytical grade.

Assessment of antibacterial activity of crude dye and components of *A. nobilis*

***Qualitative evaluation*:** Disc diffusion screening was done according to AATCC 30. Qualitative evaluation of B was carried out with the objective to detect its bacteriostatic activity. Dye solution was inoculated onto the filter paper disc of 15mm diameter in different concentrations of 5ppm, 10ppm, 50ppm and 100ppm. Additional discs containing same amount of methanol were also prepared and served as control. Discs were then left in air to evaporate the solvent. Discs containing the dye were placed onto the inoculated agar plates with sufficient space between the discs and were pressed lightly to ensure close contact with the surface. Plates were incubated at 37°C for 24 hours in an incubator. After incubation, the results were evaluated for the size of zone of inhibition produced around the sample. The contact area was also examined for any signs of bacterial growth. In all the cases, duplicate specimens were tested. The average width of a zone of inhibition on either side of the filter disc was calculated using equation 1:

$$W = (T - D)/2 \quad \text{(1)}$$

- where, W = width of clear zone of inhibition in mm
- T = total diameter of test specimen and clear zone in mm
- D = diameter of the test specimen in mm

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***Quantitative evaluation*:** The methanol stock solution of *A. nobilis* crude extract and the components (B, C, D and S) were evaluated for the degree of their antibacterial activity using AATCC 100 method. Different concentrations of the test specimen ranging from 5ppm to 200ppm were dispersed in 10ml Luria broth. 20µl of the test organism with the count of 10^6cfu/ml was then inoculated in the liquid culture medium (Luria broth). The flasks were capped, placed in the shaker and shaken at 200rpm at 37°C for 24 hours. An incubator of rotary shaker (Metrex Scientific Industries Pvt. Ltd., India) was used for incubation and growth of bacteria. Inoculated Luria broth with concentration of methanol similar to that of the test specimen was used as the positive growth control. At the predetermined time, 20µl inocula was finally taken from the test tube after serial dilutions and spread on the preset agar plates. The plates were incubated again at 37°C for 24 hours. After incubation of the plates, the number of viable cells (bacterial colonies) were counted manually using a colony counter (Yorco, India) and the results after multiplication with the dilution factor were expressed as mean colony forming units (CFU) per ml after averaging the triplicate counts. The bacterium reduction percentage (BR%) was determined by using equation 2:

$$BR\ \% = (A-B)/A \times 100 \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

- where, A= bacterial colonies for the control after 24 hours incubation time
- B= bacterial colonies for the sample after 24 hours incubation time

Assessment of antibacterial activity of dyed fabrics: Textile substrates (cotton, wool, silk, nylon, polyester and acrylic) dyed with 0.5% and 5% shade of A and B were assessed for their antimicrobial activity.

Qualitative assessment: Dyed fabrics were qualitatively assessed for their antimicrobial activity using AATCC 30. Undyed samples were taken as positive control.

Quantitative assessment: Quantitative assessment of the antibacterial activity of the dyed samples was done by colony counting method. Rectangular dyed (2inch×1inch) sample swatches were exposed to 20µl of bacterial inoculums containing 10^8 cfu/ ml of bacteria using the modified agar-plate method as described by Purwar (2004). After incubation, the bacterial colonies were counted and BR% was calculated.

Durability of antibacterial activity of dyed fabrics: Durability of the antibacterial activity of the dyed fabrics to light and washing was also assessed.

Durability to laundering: The dyed fabrics were subjected to laundering as per AATCC Test Method 61-2007 1A. Specimens in this accelerated laundering test are tested under appropriate conditions of temperature, detergent solution, bleaching and abrasive action. Each washing cycle is equivalent to five hand or home launderings. Details of this procedure are in Table 1. The fabrics were then rinsed with water and dried. The fabrics were then tested for antibacterial activity by procedure as discussed above.

***Durability to light*:** The dyed fabrics were subjected to light exposure for 180 minutes as per AATCC Test Method 16-2004. Antibacterial activity of light exposed samples was then quantitatively determined by procedure as described above.

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RESULTS

Antibacterial activity of dye solutions*:* The crude dye extract of *A. nobilis* and the separated components of the plant studied, revealed the presence of bioactive properties. Results of the disc diffusion method of B are summarized in Table 2. MIC values of the crude dye extract and the separated components against *S. aureus* and *E. coli* has been depicted in Table 3.

Antibacterial activity of the dyed fabrics: Having studied the antimicrobial activity of dyes in solution, the next step was to assess their effectiveness on dyed fabrics. Qualitative and quantitative investigations were carried out on textile substrates - cotton, wool, silk, nylon, polyester and acrylic dyed with 0.5% and 5% shade of A and B. The results of disc diffusion method showed that none of the dyed fabrics showed a zone of inhibition against *S. aureus* or *E. coli* at the test concentrations. Antibacterial activity of dyed fabrics was then quantitatively assessed to determine the percentage reduction in bacterial populations in liquid media. MIC of the dyed fabrics against the tested bacterial organisms is shown in Table 4 and 5.

Durability of antimicrobial property of dyed fabrics to light and washing: Table 6 and 7 depicts the durability of antimicrobial activity of fabrics dyed with 5% shade of A and B after repeated home launderings against *S. aureus* and *E. coli* respectively. Table 8 and 9 depicts the durability of antimicrobial activity of fabrics dyed with A and B to light.

DISCUSSION

Disc diffusion method carried out on B to detect its antibacterial activity showed a zone of inhibition of 1mm against *S. aureus* at a concentration of 10ppm. The increase in concentration of the dye to 100ppm led to an increased inhibition zone reflected by enhanced diameter of 4mm against *S. aureus* and 3mm against *E. coli*. In addition, there was a complete lack of growth under the discs at 5ppm concentration indicating that the compound is bactericidal in nature and not bacteriostatic (Singh, et al., 2005). Bigger and clearer zones of inhibition were obtained against *S. aureus* as compared to *E. coli*. Similar results have also been reported by Brigham, et al. (1999), Naz, et al. (2006) and Dhandapani and Sarkar (2007).

The quantitative assessment of the antimicrobial activity revealed that A and B are the most potent bactericides. Since B is the most prominent constituent (25%) of A, it is expected that the two would show a similar behavior. D is the least effective as a bactericide. The two samples of shikonin (D and S) showed differing antimicrobial activity. This could be due to the fact that the laboratory prepared sample of shikonin (D) is a mixture of several components and is therefore less pure (Arora, et al., in press). In all the cases, the components showed higher activity against *S. aureus* (5ppm for A, B) as compared to the Gram negative bacteria, *E. coli* (10ppm). Similar results have been reported by other researchers working with natural dyes (Brigham, et al., 1999; Naz, et al., 2006; Dhandapani and Sarkar, 2007).

Two mechanisms have been proposed for the antimicrobial activity of naphthoquinones based on structure property studies.

(i) Oxidative stress: The naphthoquinone molecule which is readily reduced by flavoenzymes to semiquinone radical anion is further converted to hydroquinone by quinone oxidoreductase. Once the semiquinone radical anion reacts with oxygen, it can generate superoxide anions, which are known to cause the oxidative stress in microbes. This is a process that can cause damage to membranes, proteins and also

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DNA (Ames, et al., 1986; Friedrich, 1988; O'Brien, 1991; Cho, et al., 1997; Gao, et al., 2000; Boelsterli, 2003; Boudalis, et al., 2008; Valavanides, 2009).

(ii) Alkylation: The second process is that of alkylation. Naphthoquinones are activated inside the microbial cells and become covalently attached to the cellular nucleophiles such as proteins and basic parts of DNA, often leading to inactivation of proteins and loss of function. Probable targets in the microbial cell are surface-exposed adhesions, cell wall polypeptides and membrane bound enzymes thus rendering the substrates unavailable to the micro-organisms (You, et al., 1998; Cowan, 1999; Chen, et al., 2002; Naz, et al., 2006; Lenta, et al., 2007; Dhandapani and Sarkar, 2007).

The dyed fabrics didn't show any zone of inhibition against both the bacteria. This may be because the dye is bound closely to the substrate and hence, it is no longer soluble and therefore, does not leach out to give a zone. At the same time no growth was witnessed under or on the discs. Absence of growth on the substrates affirmed the antimicrobial activity of the dyed substrates. The results of the quantitative assessment of the dyed fabrics interestingly showed that the reduction percentage of bacteria varies from no activity (0%) to maximum activity (100%) on different textile fibres. In general, the results obtained are similar for the two components.

Bactericidal property of dyed polyester is highly sensitive to concentration of the dye used; jumping from 0% to 100% reduction against *S. aureus* and ~ 80% reduction against *E. coli* with a tenfold increase in dye concentration. Nylon fabric showed no activity at both the dye concentrations, while acrylic shows 100% activity at both the concentrations used. Looking at the results obtained for natural fibres - cotton, wool and silk, similar differentiation in microbial inhibitory activity was observed. While wool and silk showed nearly 100% activity at 5% concentration of dye against both the microbes, cotton showed no activity at all.

Two reasons can be proposed for such fibre specific differences in antimicrobial activity. The first relates to the chemical interactions between the dye and fibre. Polyester being hydrophobic, non polar fibre, has high affinity for the similarly hydrophobic dye molecule. The small molecules of dye dissolve in it and are held there by hydrophobic forces of attraction. The molecule therefore remains chemically free and unchanged in the fibre. Nylon being an ionic fibre can react with the dye. This is happening in this case and is evident from the fact that while the dye gives a pink colour on polyester, it gives a blue colour on nylon indicating that nylon may be reducing the dye (Arora, et al., 2012). To check this hypothesis an experiment was conducted where the solution of A was buffered at pH 10. It was seen that the colour of solution changed from red to blue. The antimicrobial activity of this solution was assessed and the MIC was found to be 75ppm as compared to 5ppm against *S. aureus* for the non-reduced dye solution. Similarly, for *E. coli*, the MIC increased to 90ppm as compared to 10ppm of the original solution. This indicated that the antimicrobial activity of the dye is getting severely decreasing in the reduced form and therefore, it does not show any activity on nylon fibre.

Natural fibres also showed a difference in activity. This may be attributed to the dye- fibre affinity which also determines the amount of dye taken up by a fibre from the dye bath. Wool and silk being amphoteric in nature, take up the dye easily building up a large concentration in the fibre. As wool is more amorphous it takes up more dye than silk. Cotton on the other hand, due to its highly crystalline nature can only be dyed with dyes having a long, linear and planar molecule. Since the

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compounds used in this study do not have such a structure, cotton has no affinity for these dyes and therefore, remains largely undyed and thus, does not show any antimicrobial activity. Thus, it can be inferred that the concentration of dye used as well as the nature of fibre significantly affects the antimicrobial properties of textiles dyed with *Arnebia* extracts. This is in accordance with results reported earlier, Gupta, et al. 2004; 2005.

Durability of antimicrobial activity of fabrics dyed with 5% shade of A and B respectively after repeated home launderings showed that the fabrics retained almost 90% - 98% of their antibacterial activity up to 25 cycles of laundering against both the bacteria. The results demonstrated that A and B had strong affinity for wool, silk, acrylic and polyester. Such affinity was considered to be due to the ionic and Van der Waals attractions between the dye and wool, silk and acrylic. On the other hand, the compact structure of polyester did not allow the dye to come out during the launderings which was carried out at 45°C. Thus, its antibacterial activity was also retained. Cotton and nylon did not show any antimicrobial activity. It was observed that all the dyed fabrics showed similar results against both the bacteria.

Fabrics dyed with A and B dye solutions were exposed to light as per AATCC Test Method 16 – 2004. The dyed fabrics showed poor resistance to light and the colour faded away after 2 hours exposure. The faded fabrics still showed antibacterial activity against *E. coli* and *S. aureus* though a substantial reduction of 33% – 40% in antibacterial activity was observed after the exposure. This may be because these naphthoquinones based dyes are susceptible to photo-oxidation on exposure to light. The dyed fabrics showed similar results against both the bacteria.

CONCLUSIONS

The study showed that the naphthoquinone colourants of *A. nobilis* can be used to impart antimicrobial properties to textiles. The dye and its components showed excellent antimicrobial activity against both *S. aureus* and *E. coli*. Among the fabrics dyed with 5% shade of crude dye and alkannin β, β-dimethylacrylate, wool, silk and acrylic showed 100% activity against both microbes. Polyester showed 100% activity against *S. aureus* and ~ 80% activity against *E. coli w*hile nylon and cotton showed no antimicrobial activity. The dyed textiles retained their antimicrobial activity upto 25 home launderings whereas 35% – 40% reduction was observed in activity when the substrates were exposed to light for 2 hours.

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Table-1: Specifications of accelerated laundering as per AATCC Test Method 61 – 2007 1A.

Parameter	Quantity
Specimen size	2.0 × 4.0inch
Temperature	40°C ± 2°C
Total liquor volume	200ml
% detergent of total volume	0.37
Steel balls	10
Time	45 minutes

Table-2: Bioassay of Component B of *Arnebia nobilis* extract by disc diffusion method.

Concentration (ppm)	Zone of inhibition (mm)	
	S. aureus	***E. coli***
5	0.0	0.0
10	1.0	1.0
50	3.5	2.0
100	4.0	3.0

Table-3: Minimum Inhibitory Concentration of dyes against *S. aureus* and *E. coli* .

Sample	***S. aureus* (ppm)**	***E. coli* (ppm)**
A	5	10
B	5	10
C	20	40
D	200	200
S	100	100

- A: Crude dye extract
- B: Component B
- C: Component C
- D: Component D
- S: Standard shikonin

Table-4: Bacteria reduction percentage (BR%) of dyed fabrics against *S. aureus*.

Sample	A		B	
	0.5% shade	**5% shade**	**0.5% shade**	**5% shade**
Polyester	0	100	11.6	99.9
Nylon	0	0	0	0
Wool	99.9	99.9	99.9	99.9
Silk	54	100	67.5	100
Acrylic	100	100	100	100
Cotton	0	0	0	0

- A: Crude dye extract of *A. nobilis*
- B: Component B (alkannin β, β- dimethylacrylate) of *A. nobilis*

Table-5: Bacteria reduction percentage (BR%) of dyed fabrics against *E. coli*.

Sample	A		B	
	0.5 % shade	**5 % shade**	**0.5 % shade**	**5 % shade**
Polyester	0	77	11.6	75
Nylon	0	0	0	0
Wool	99.9	99.9	99.9	99.9
Silk	54	100	67.5	100
Acrylic	100	100	100	100
Cotton	0	0	0	0

- A: Crude dye extract of *A. nobilis*
- B: Component B (alkannin β, β- dimethylacrylate) of *A. nobilis*

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Table-6: Durability of antimicrobial activity of fabrics to wash against *S. aureus*.

Sample	A (BR%)		B (BR%)	
	Original	25 washes	Original	25 washes
Polyester	100	91	99.9	90
Wool	99.9	98	99.9	98
Silk	100	89	100	89
Acrylic	100	98	100	98
Cotton	0	0	0	0
Nylon	0	0	0	0

- A: Crude dye extract of *Nobilis*
- B: Component B (alkannin β, β- dimethylacrylate) of *Nobilis*

Table-7: Durability of antimicrobial activity of fabrics to wash against *E. coli*.

Sample	A (BR%)		B (BR%)	
	Original	25 washes	Original	25 washes
Polyester	77	71	75	67.5
Wool	99.9	98	99.9	98
Silk	100	89	100	89
Acrylic	100	98	100	98
Cotton	0	0	0	0
Nylon	0	0	0	0

- A: Crude dye extract of *Nobilis*
- B: Component B (alkannin β, β- dimethylacrylate) of *A. nobilis*

Table-8: Durability of antimicrobial activity of fabrics to light against *S. aureus*.

Sample	A (BR%)		B (BR%)	
	Unexposed	Exposed	Unexposed	Exposed
Polyester	100	50	99.9	60
Wool	99.9	63	99.9	63
Silk	100	45	100	45
Acrylic	100	63	100	63
Cotton	0	0	0	0
Nylon	0	0	0	0

- A: Crude dye extract of *Nobilis*
- B: Component B (alkannin β, β- dimethylacrylate) of *A. Nobilis*

Table-9: Durability of antimicrobial activity of fabrics to light against *E. coli*.

Sample	A (BR%)		B (BR%)	
	Unexposed	Exposed	Unexposed	Exposed
Polyester	77	50	75	60
Wool	99.9	63	99.9	63
Silk	100	45	100	45
Acrylic	100	63	100	63
Cotton	0	0	0	0
Nylon	0	0	0	0

- A: Crude dye extract of *Nobilis*
- B: Component B (alkannin β, β- dimethylacrylate) of *A. Nobilis*

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