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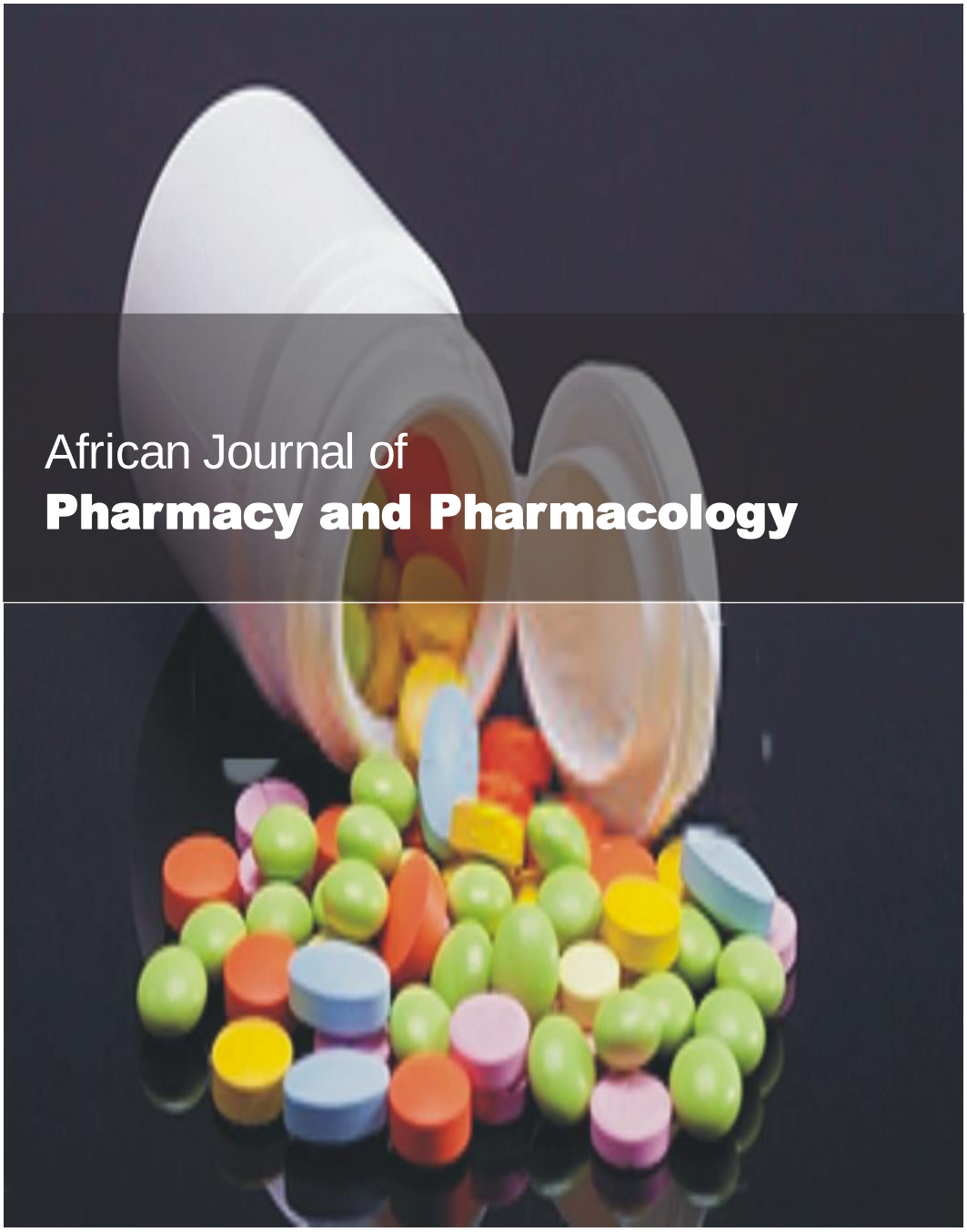

African Journal of
Pharmacy and Pharmacology

15 March 2018
ISSN 1996-0816
DOI: 10.5897/AJPP
www.academicjournals.org

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African Journal of Pharmacy and Pharmacology

Table of Contents: Volume 12 Number 10 15 March, 2018

ARTICLE

academicJournals

Vol. 12(10), pp. 121-129, 15 March, 2018
DOI: 10.5897/AJPP2018.4642
Article Number: F163A6556266
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

In vitro antimicrobial activities of *Plocamium rigidum* and *Plocamium cornutum* from the Namibian coast line

Anthony Ishola[1*], Michael Knott[1] and Jane Misihairabgwi[2]

[1]Department of Pharmaceutical Chemistry, School of Pharmacy, University of Namibia. Private Bag 13301, Windhoek, Namibia.
[2]Department of Biochemistry and Microbiology, School of Medicine, University of Namibia, Private Bag 13301, Windhoek, Namibia.

Received 12 January, 2018; Accepted 6 February, 2018

The aim of this study was to determine suitable extraction solvents for antimicrobial compounds and the antimicrobial activity of two algal species, *Plocamium cornutum* and *Plocamium rigidum* collected from the coastline of Namibia. Samples were collected at low tide from the intertidal area of the coastline at Lüderitz and Henties Bay. The samples were collected about 5 to 10 cm under water by hand and placed in a sealable polythene bag and refrigerated at -20°C. Dried algae extracts were reconstituted in distilled water, hexane, dichloromethane, ethanol, methanol and chloroform, respectively and tested *in vitro* for antimicrobial activity using the Kirby Bauer disc diffusion method against 12 pathogens (*Escherichia coli, Staphylococcus aureus, Staphylococcus saprophyticus, Pseudomonas aeruginosa, Streptococcus pyogenes, Proteus mirabilis, Listeria monocytogenes, Shigella sonnei, Salmonella* species*, Enterococcus faecalis, Candida albicans* and *Staphylococcus epidermidis*). Screening confirmed that water extracts showed no activity against all the pathogens as the extracts were insoluble in water. The *Plocamium* extracts in the remaining solvents showed varying degrees of antimicrobial activity. Both dichloromethane and methanol extracts reconstituted in chloroform showed the greatest activity amongst the five different solvents that were used. Ampicillin (10 µg/ml) showed no antimicrobial activity against *S. epidermidis* whilst a zone of inhibition of 6.26±0.07 mm was recorded for 10 µg/ml of *P. cornutum* extract reconstituted in chloroform. An ethanolic extract of *P. rigidum* showed a zone of inhibition of 6.35±0.25 mm against *L. monocytogenes* while the standard ampicillin had no activity. Extracts of *P. rigidum* in ethanol and *P. cornutum* in chloroform are evidently potential lead candidate antibiotics *in vitro* against *L. monocytogenes* and *S. epidermidis*, respectively.

Key words: Antimicrobial activity, *Plocamium cornutum*, *Plocamium rigidum*, *Listeria monocytogenes*.

INTRODUCTION

Due to emerging resistance of pathogenic microorganisms to existing antibiotics and the fast spreading of resistant microorganisms, there is an increasing need for new antibiotics. In marine

*Corresponding author. E-mail: aishola@unam.na.

Table 1. Common diseases associated with the pathogens used in this research.

Name	Type of pathogen	Disease
Escherichia coli	Gram negative	Diarrhoea
Staphylococcus aureus	Gram positive	Sinusitis, skin infection
Staphylococcus saprophyticus	Gram positive	Urinary tract infection and cystitis
Pseudomonas aeruginosa	Gram negative	Sepsis syndromes, hospital acquired infections
Sreptococcus pyogenes	Gram negative	Tonsillitis, sinusitis
Proteus mirabilis	Gram negative	Kidney stones, renal failure
Listeria monocytogenes	Gram positive	Listeriosis
Shigella sonnei	Gram negative	Invade epithelial lining of the colon, diarrhea
Salmonella typhi	Gram negative	Food poison
Enterococcus faecalis	Gram positive	Urinary tract infection, endocarditis, meningitis
Candida albicans	Fungus (yeast)	Candidiasis (mouth or genital itching)
Epidermidis	Gram positive	Biofilms (catheters)

environments, competition for space and nutrients led to the evolution of antimicrobial defence strategies. This includes the production of chemically active metabolites in their surroundings which act as an aid to protect themselves against other settling organisms, maintenance of unfouled surfaces, deterrence of predation, the ability to successfully reproduce, protection from UV radiation and as allelopathic agents (Taskin et al., 2012; Chakraborthy et al., 2010). Marine algae are one of the largest producers of biomass in the marine environments and are a rich source of structurally novel biologically active metabolites (El-Din and El-Ahwany, 2016). Therefore, they offer a rich source of potentially new drug leads.

Algae metabolites have great industrial potential and accessibility, and thus they have attracted attention for health and cosmetic applications. The use of microalgae and their derivatives in applications to combat skin aging, as well as for depigmentation and antimicrobial applications in the cosmetic industry is wide spread (Wang et al., 2015). A wide range of metabolites, such as antioxidants, anti-inflammatory agents, alginates, polysaccharides, and carotenoids, have been investigated for cosmeceutical preparations. The antimicrobial properties of marine algae have been known since ancient times and well documented in recent years (Patra et al., 2008). Algae are thus a source of raw materials for one of the most promising and profitable sectors of the biotechnology industry. Phycocolloid substances from marine algae such as alginate, carrageenan and agar have been used globally for decades in medicine and pharmacy. Thus, they are of interest for potential use in cosmetic products (Patra et al., 2008). Four species of Algerian marine algae were tested for anti-fungi properties and results showed that they had fungi inhibiting effects (Patra et al., 2008). Algal materials collected from the Red Sea Coast of Jeddah inhibited the growth of *Enterococcus faecalis* (11 mm) but no activity was recorded for water extracts (Al-Saif et al., 2014).

Marine algae provide a rich source of structurally diverse secondary metabolites some of which have marked antimicrobial activity against marine pathogens. The bactericidal agents found in algae include amino acids, terpenoids, phlorotannins, acrylic acid, phenolic compounds, steroids, cyclic polysulphides and fatty acids. Watson and Cruz-Rivera (2003) reported that 54 seaweeds were tested *in vitro* for antimicrobial activity and a staggering 95% of the extracts showed activity against different pathogens. Bromophenol compounds have been frequently encountered in various marine algae including red and brown algae. Red algae of family Rhodomaceae are especially known as a rich source of bromophenols (Oh et al., 2008). Some of these compounds which were previously isolated from the family exhibited a wide spectrum of pharmacological activities such as enzyme inhibition, cytotoxic, antioxidant, feeding deterrent, anti-inflammatory, and antimicrobial activities (Williamson and Carughi, 2010). Taskin (2012), studied the inhibitory activities of various organic extracts of algae against various fish pathogenic bacteria and their results confirmed the possible use of some marine algae as a source of antimicrobial compounds

In this study, organic crude extracts from *Plocamium* species, a red marine algae from the coastline of Namibian, were studied for their potential inhibitory activities against common pathogens. Twelve pathogens selected for this study are important in our everyday life as they are common causes of a variety of human diseases (Table 1).

The ability of marine algae to produce metabolites of potential interest has been extensively documented. The antimicrobial activity of marine algae may be influenced by some factors such as the habitat, the season of algal collection, different growth stages of plant and experimental methods (Al-Saif et al., 2014). Although a variety of solvents have been employed in screening

marine algae for antimicrobial activity, it is still uncertain what kinds of solvents are most effective and suitable for the extraction of secondary metabolites from marine algae (Manivannan et al., 2011). El-Din and El-Ahwany (2015) also reported that antimicrobial activity depends on the solvents used for extraction. It was found that benzene and diethyl ether were suitable solvents for extracting various antibiotic compounds. However, extracts obtained with acetone, ethyl alcohol and ether showed higher antimicrobial activity than extracts from chloroform (Manivannan et al., 2011). In a similar study, Sasidharan et al. (2009) found that chloroform exhibited the strongest activity, which is consistent with the findings of (El-Din and El-Ahwany, 2015).

There is a dearth of information concerning the antimicrobial activities of Namibian marine algae. Antimicrobial potential of Namibian marine algae are unexplored despite the availability of marine algae in the coastal areas of the country. This study therefore aimed to screen for potentially bioactive metabolites from Namibian marine algae and test them for antimicrobial activity. This would be beneficial to the development of Namibian medicine if a novel solution was found to common microbial infections.

MATERIALS AND METHODS

Sample collection

Plocamium spp. were collected during low tide along the coastline of Namibia from Henties Bay and Lüderitz, where they are abundant in intertidal, shallow and coastal estuaries. The samples were collected about 5 to 10 cm under water by hand and placed in a sealable polythene bag and refrigerated at -20°C. Collections were done twice from the same areas. The coordinates of Lüderitz and Henties Bay are 26° 38' 53" S, 15° 9' 34" E and 22° 7' 0"S, 14° 17' 0" E, respectively. As the samples co-exist with other settling organisms, they were first washed in seawater and then in fresh water to remove sand, epiphytes and other necrotic parts. The identification of *Plocamium cornutum* and *Plocamium rigidum* was done on the basis of their morphological characteristics, including colour and the arrangement of the branchlets. The final identification was done at the Marine and Fisheries Department of the University of Namibia by visual appearance and standard collection guides (Lluch, 2002). The samples were transported to the laboratory in polythene bags under ice and were frozen at -87°C for future analyses.

Test microorganisms

Extracts of *P. rigidum* and *P. cornutum* were investigated to evaluate their antimicrobial activity against five Gram positive human pathogens (*Staphylococcus aureus, Staphylococcus saprophyticus, Listeria monocytogenes* and *E. faecalis*), 6 Gram negative human pathogens (*Escherichia coli, Pseudomonas aeruginosa, Streptococcus pyogenes, Proteus mirabilis, Shigella sonnei* and *Salmonella*) and a fungi, *Candida albicans* using a disc diffusion method. The 12 pathogens used in this study were obtained from the Department of Biochemistry and Microbiology, School of Medicine, University of Namibia.

Preparation of marine algae extracts

Frozen samples of marine algae (33 g) were soaked in extraction solvent (MeOH:DCM 1:1 v/v) for 48 h at room temperature (±26°C). The extracts were then decanted into a 250 ml beaker. The resulting extracts were filtered through Whatman No. 1 filter paper and concentrated using a rotary evaporator (model RE 100; Bibby Sterilin Ltd). The concentrated extracts were then dissolved in 100 ml of extraction solvent and transferred to a separating funnel. Two layers of immiscible liquids were formed in the separating funnel, of which the upper layer consisted of MeOH extract which form the polar fraction and the lower layer composed of DCM extracts which is the non-polar fraction. The MeOH and DCM layers were carefully decanted into pre-weighed glass vials. The extracts were left to dry in a laminar airflow cabinet. The dried extracts were weighed and placed in a freezer at a temperature of about -80°C. DCM and MeOH extracts of *Plocamium* spp. were reconstituted in six different solvents to yield solutions of 1 mg/ml in water, hexane, dichloromethane, ethanol, methanol and chloroform (H_2O, C_6H_{14}, DCM, EtOH, MeOH and CH_3Cl) to evaluate the antimicrobial potential of the different solvent extracts.

Microbial inoculum preparation

All media used in the study were supplied from Hi Media laboratories, India. Standard ampicillin antibiotic susceptibility discs were obtained from SRL chemicals Ltd., India. All media were prepared in deionised water and autoclaved at 121°C for 15 min prior to use according to the manufacturers' instructions. Twelve selected pathogens were cultured on nutrient agar plates. The plates were incubated at 35 to 37°C in an incubator (Scientific series 2000, L. digital incubator; model 286) for 24 h. Colonies of each of the selected twelve bacteria were inoculated into Tryptone Soya Broth in 2 ml culture tubes. The culture tubes were left to incubate at 35 to 37°C for 24 h.

Determination of antimicrobial activity of *Plocamium* spp.

The antimicrobial activity of the *Plocamium* extract was carried out using the Kirby Bauer disc diffusion method (Arullappan et al., 2009). The DCM and methanol extracts were tested separately against 12 pathogens and the tests were run in triplicate and average inhibition zones were recorded using Vernier callipers. The negative controls consisted of six different sterile solvents (H_2O, C_6H_{14}, DCM, EtOH, MeOH and CH_3Cl) used to dissolve the extracts. The culture solutions were used to impregnate the diffusion disc used for antimicrobial screening.

Twelve petri dishes with Mueller-Hinton agar were impregnated with a different bacteria and each dish was divided into three sectors. Discs soaked in different extracts in their respective solvents were placed in different sectors of the petri dish along with both negative and positive controls. Three petri dishes with the same extract and controls (triplicate) were incubated at 37°C for 24 h. The zones of inhibition were measured after 24 h.

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined for all extracts that showed antimicrobial activities in the screening phase. This was carried out using a modified procedure from (Peng et al., 2010). Each algal extract was serially diluted to yield 800, 600, 400, 200, 100, 20 and 10 µg/ml. Filter paper discs were placed in each of the diluted extract to absorb their respective solvent for 15 min and allowed to dry. The discs were respectively placed onto the

Table 2. Screening DCM extracts reconstituted in different solvents for antimicrobial activity against pathogens.

Solvents	Algal species	*E. coli*	*P. aeruginosa*	*S. sonnei*	*S. pyogenes*	*P. mirabilis*	*E. faecalis*	*L. monocytogenes*	*Salmonella*	*S. epidermidis*	*S. saprophyticus*	*C. albicans*	*S. aureus*
$CHCl_3$	*P. rigidum*	+++	-	+	-	-	+	-	-	+	-	-	-
	P. cornutum	-	-	-	+	+	+	-	-	+	-	-	-
C_6H_{14}	*P. rigidum*	+	-	+++	-	-	-	++	-	-	-	-	-
	P. cornutum	+	-	++	-	-	-	+	-	-	-	-	-
DCM	*P. rigidum*	-	+	+	+	+	+	++	-	-	-	-	-
	P. cornutum	-	+	+	+	+	+	+	-	+	-	-	-
EtOH	*P. rigidum*	-	-	+++	+	-	++	-	+	-	-	-	++
	P. cornutum	-	++	+	+	-	++	-	-	+	-	-	+
Water	*P. rigidum*	-	-	-	-	-	-	-	-	-	-	-	-
	P. cornutum	-	-	-	-	-	-	-	-	-	-	-	-

Inhibition zone diameters: -, < 6 mm; +, 6 – 10 mm; ++, 10 – 15 mm; +++, 15 – 20 mm. Chloroform ($CHCl_{3)}$, Hexane ($C_6H_{14)}$, Ethanol (EtOH), Dichloromethane (DCM).

plates containing each of the twelve selected bacteria. Standard ampicillin antimicrobial susceptibility test discs of 10 µg/ml and blank solvent discs served as the positive and negative controls, respectively. Different discs with different concentrations along with positive and negative controls were placed in their respective divisions and labelled on the petri dishes. The petri dishes were prepared in triplicates. The plates were incubated at 37°C for 24 h. MIC was defined as the lowest concentration at which no visible growth was observed; this indicates the presence of antimicrobial activity.

Statistical analysis

Means of triplicate analysis were calculated and data was expressed as mean ± standard deviation (SD) and analysed using SPSS 22 software. A Shapiro test was used to determine the normality of MIC. Kruskal-Wallis test was then used to identify significant differences in the MIC among the treatment groups ($p<0.05$). Dun-Bonferroni *post hoc* statistical test was used for pairwise comparison of the treatment groups.

RESULTS

Antimicrobial screening

Water extracts showed no antimicrobial activity against all the pathogens. The antimicrobially active algae extracts were probably not soluble in water. The remaining solvents had varying degrees of antimicrobial activity as shown in Tables 2 and 3 for DCM and MeOH extracts, respectively.

Table 2 highlights that DCM extracts of *P. rigidum* and *P. cornutum* reconstituted in chloroform and ethanol showed high antimicrobial activity (+++) against, *E. coli, S. sonnei* and *S. aureus*. Similar extracts only demonstrated moderate antimicrobial activity (++) against *P. aeruginosa*, *E. faecalis*, *L. monocytogenes* and *S. sonnei* when reconstituted in ethanol, dichloromethane and hexane. DCM extracts of *P. rigidum* and *P. cornutum* reconstituted in chloroform, ethanol and other solvents showed only traces of antimicrobial activity (+) or no activity against the pathogens.

Table 3 shows that the antimicrobial activity of MeOH extracts of algae reconstituted in chloroform, hexane, dichloromethane and ethanol showed less activity against the selected pathogens, compared to DCM extracts. MeOH extracts of *P. rigidum* and *P. cornutum* reconstituted in chloroform, hexane and ethanol showed high antimicrobial activity (+++) against, *E. coli* and *S. sonnei*. Similar extracts only demonstrated moderate antimicrobial activity (++)

Table 3. Screening methanol extracts reconstituted in different solvents for antimicrobial activity against pathogens.

Solvents	Algal species	*E. coli*	*P. aeruginosa*	*S. sonnei*	*S. pyogenes*	*P. mirabilis*	*E. faecalis*	*L. monocytogenes*	*Salmonella*	*S. epidermidis*	*S. saprophyticus*	*C. albicans*	*S. aureus*
$CHCl_3$	*P. rigidum*	+++	-	+	-	-	+	-	-	+	-	-	-
	P. cornutum	-	-	-	-	-	-	-	++	-	+	-	+
C_6H_{14}	*P. rigidum*	+	-	+++	-	-	-	+	-	-	++	-	-
	P. cornutum	-	-	-	-	-	-	-	-	-	-	-	-
DCM	*P. rigidum*	-	-	-	-	-	-	-	-	-	-	-	-
	P. cornutum	-	-	-	-	-	-	-	-	-	-	-	-
EtOH	*P. rigidum*	-	+	+++	+	+	++	++	+	-	+	+	+
	P. cornutum	-	++	-	-	-	-	+	-	-	-	-	-
MeOH	*P. rigidum*	-	-	-	+	+	+	+	-	+	+	-	+
	P. cornutum	-	-	-	-	-	-	-	-	-	+	-	+
Water	*P. rigidum*	-	-	-	-	-	-	-	-	-	-	-	-
	P. cornutum	-	-	-	-	-	-	-	-	-	-	-	-

Inhibition zone diameters: -, < 6 mm; +, 6 – 10 mm; ++, 10 – 15 mm; +++, 15 – 20 mm. Chloroform ($CHCl_3$), Hexane (C_6H_{14}), Ethanol (EtOH), Dichloromethane (DCM).

against, *E. faecalis*, *L. monocytogenes, Salmonella* and *S. saprophyticus* when reconstituted in ethanol, chloroform and hexane.

Minimum inhibitory concentration of algal extracts

Evaluation of antimicrobial activity of the algal extracts was recorded in Tables 4, 5 and 6. Of the six solvents used to reconstitute extracts from dichloromethane and methanol for antimicrobial activity, water extracts showed no activity against any of the twelve pathogens used in the test. This is in agreement with the results obtained of Al-Saif et al. (2014). The other algal extracts reconstituted in C_6H_{14}, DCM, EtOH, and CH_3Cl demonstrated varying degrees of inhibitory activity against the test pathogens. Chloroform proved to the solvent of choice, as it was observed to have significantly higher inhibitory activity against tested pathogens ($p<0.05$). Ampicillin (10 µg/ml) showed no activity against *S. epidermidis* in DCM extract of *P. rigidum* reconstituted in chloroform.

Antimicrobial activity of DCM extracts of algae reconstituted in chloroform

DCM extracts reconstituted in chloroform showed prominent antimicrobial activity (15 to 20 mm) against *E. coli* but only traces of antimicrobial activity (6 to 10 mm) against *E. faecalis, S. Epidermidis* and *S. sonnei* species in DCM extracts of *P. rigidum* and *P. cornutum.*

S. saprophyticus and *C. albicans* proved resistant to all the extracts of DCM reconstituted in chloroform and ethanol. *P. mirabilis, S. pyogenes* and *S. sonnei* also showed traces of activity (6 to 10 mm) in all DCM extracts. DCM extracts of sample *P. rigidum* demonstrated a ZOI of 10 to 15 mm against *L. monocytogenes*.

DCM extracts of *P. rigidum* reconstituted in hexane showed activity of 10 to 15 mm against *L. monocytogenes* and *S. sonnei* but only traces of antimicrobial activity against *E. coli.*

DCM extracts of *P. rigidum* reconstituted in EtOH showed high antimicrobial activity (15 to 20 mm) against *S. sonnei* and *S. aureus* but moderate antimicrobial activity (10 to 15 mm) against *E. faecalis*. The extract showed only

Table 4. MIC of DCM extracts of *Plocamium* species reconstituted in chloroform against pathogens.

Extract	Conc. (µg/ml)	Inhibition zone (mm)				
		Gram (+ve) pathogens		Gram (-ve) pathogens		
		E. faecalis	*S. epidermidis*	*E. coli*	*S. sonnei*	*S. pyogenes*
P. rigidum	5	-	-	-	-	-
	10	7.41 ± 0.15	6.21±0.19	7.42±0.15	-	-
	20	7.55 ±0.28	6.41±0.15	8.06±0.23	6.88±0.1	-
	100	8.15 ± 0.2	6.55±0.23	8.62±0.22	7.38±0.08	-
	200	8.33 ± 0.43	6.71±0.25	9.86±0.22	7.45±0.12	-
	400	8.42 ± 0.35	6.84±0.23	9.67±0.35	7.86±0.24	-
	600	9.05 ± 0.6	6.92±0.21	10.49±0.2	9.27±0.16	-
	800	9.64 ± 0.63	7.81 ±0.45	13.35±0.27	11.26±0.28	-
P. cornutum	5	-	-	-	-	-
	10	-	6.26±0.07	-	-	-
	20	-	6.43±0.08	-	-	-
	100	-	6.88±0.08	-	-	-
	200	-	7.16±0.03	-	-	-
	400	-	7.55±0.12	-	-	6.88±0.11
	600	-	7.75±0.17	-	-	7.75±0.08
	800	8.95±0.21	8.17±0.16	-	-	8.49±0.1

Data are presented as mean values ± SD. – No inhibition.

Table 5. MIC of DCM extracts of Plocamium species reconstituted in ethanol against pathogens.

Extract	Conc. (µg/ml)	Inhibition zone (mm)					
		Gram (+ve) pathogens			Gram (-ve) pathogens		
		E. faecalis	*S. epidermidis*	*L. monocytogenes*	*E. coli*	*S. sonnei*	*S. pyogenes*
P. rigidum	5	-	-	-	-	-	-
	10	-	-	6.35±0.05	-	-	-
	20	-	-	6.37±0.05	-	-	-
	100	-	-	6.44±0.04	-	-	-
	200	-	-	6.51±0.06	-	-	-
	400	6.38±0.14	-	6.83±0.11	-	-	-
	600	6.43±0.06	-	7.55±0.13	-	9.38±0.03	-
	800	8.77±0.05	-	10.94±0.05	-	12.45±0.08	-

Data are presented as mean values ± SD. – No inhibition.

traces of inhibition against S. pyogenes and S. epidermidis. Ampicillin (10 µg/ml) showed no activity against L. monocytogenes in DCM extract of P. rigidum reconstituted in ethanol.

The results of methanol extracts of Plocamium species reconstituted in EtOH (Table 6) revealed that the algal extracts were potentially active in suppressing microbial growth of S. saprophyticus and L. monocytogenes. It was found that the ZOI of 10 µg/ml ampicillin against L. monocytogenes was 10.05 mm as compared to 10.26 mm recorded for the MeOH extract of P. rigidum reconstituted in EtOH.

The lowest extract concentration that prevents visible bacterial growth was determined for allthree extracts. *P. rigidum* showed a ZOI of 6.35±0.25 at the lowest MIC of 10 µg/ml. This could be an indication of better antibacterial activity of this extract in comparison to other extracts.

DCM extracts of *P. rigidum* reconstituted in chloroform showed a MIC of 10 µg/ml and a ZOI of 7.41 ± 0.2 mm against *E. faecalis.* The same extract with MIC of 10 µg/ml showed a ZOI of 6.21 ± 0.08 mm against *S. epidermidis* which indicates a better growth inhibition of *E. faecalis* than *S. epidermidis*. The same algal extract

Table 6. MIC of MeOH extracts of Plocamium species reconstituted in ethanol against pathogens.

Extract	Conc. (µg/ml)	Inhibition zone (mm)					
		Gram (+ve) pathogens			Gram (-ve) pathogens		
		E. faecalis	*S. saprophyticus*	*L. monocytogenes*	*E. coli*	*S. sonnei*	*S. pyogenes*
P. rigidum	5	-	0.0±0.0	0.0±0.0	-	-	-
	10	-	9.28±0.055	10.26±0.0	-	-	-
	20	-	9.89±0.034	11.05±0.07	-	-	-
	100	-	10.88±0.043	11.23±0.05	-	-	-
	200	-	11.43±0.022	11.90±0.06	-	-	-
	400	-	11.15±0.1	12.60±0.02	-	-	-
	600	-	13.57±0.09	13.25±0.05	-	-	-
	800	-	14.37±0.07	13.91±0.03	-	-	-

Data are presented as mean values ± SD. – No inhibition.

Table 7. MIC of MeOH extracts of *Plocamium* species reconstituted in ethanol against pathogens.

Extracts	Conc. (µg/ml)	Inhibition zone (mm)			
		Gram (+ve) pathogens		Gram (-ve) pathogens	
		E. faecalis	*L. monocytogenes*	*E. coli*	*P. aeruginosas*
P. cornutum	5	-	-	-	-
	10	-	-	-	9.76±0.017
	20	-	-	-	10.11±0.082
	100	-	-	-	10.38±0.02
	200	-	-	7.26±0.043	10.75±0.082
	400	-	-	8.22±0.043	11.34±0.049
	600	-	-	12.94±0.058	10.80±0.1
	800	-	-	13.00±0.041	11.52±0.1

Data are presented as mean values ± SD. – No inhibition.

demonstrated the same growth inhibition against *E. coli* at 10 µg/ml. However, as the algal concentration increases, there is a marked growth inhibition demonstrated in *E. coli* and *S. sonnei* than the gram positive *E. faecalis* and *S. epidermidis*. DCM extracts of P. rigidum reconstituted in chloroform thus has the characteristics of a broad spectrum antibiotic in vitro. Table 5 shows that DCM extracts of P. cornutum reconstituted in chloroform showed no activity at lower extract concentration against *E. faecalis* and S. pyogenes. However, weak inhibitions were recorded at 400 µg/ml. There is no activity against *E. coli* and *S. sonnei.*

DCM extracts of *P. rigidum* reconstituted in ethanolshowed pronounced activity against L. monocytogenes at low concentration (MIC of 10 µg/ml and ZOI of 6.35±0.25 mm). This result was better than the observed activity of standard ampicillin antimicrobial susceptibility test disc against the same pathogen.

Antimicrobial activity of MeOH extracts of algae reconstituted in ethanol

MeOH extracts of *P. rigidum* reconstituted in ethanol showed no activity against *E. faecalis, E. coli, S. sonnei* and *S. pyogenes.* The extracts however demonstrated strong activity against *S. saprophyticus* and *L. monocytogenes*. While standard ampicillin antimicrobial susceptibility test disc of 10 µg/ml did not inhibit the growth of *L. monocytogenes*, 10 µg/ml of *P. rigidum* reconstituted in ethanol inhibited the growth of *L.*

monocytogenes by 10.26±0.01 mm.

MeOH extracts of *P. cornutum* reconstituted in ethanol only inhibited the growth of *E. coli* and *P. aeruginosa*. Table 7 shows that the activity against *P. aeruginosa* even at low concentration of 10 µg/ml is significant as this pathogen is resistant standard ampicillin.

DISCUSSION

The data obtained in the present study indicated that chloroform was the most effective solvent for the extraction of bioactive compounds, followed by ethanol. Organic solvents always have a higher efficiency in extracting antimicrobial compounds than water as the solvent for extraction (Kamra and Bhatt, 2012). This result is consistent with literature that extracts from organic solvents give more consistent antimicrobial activity than water extracts (Kamra and Bhatt, 2012; Tiwari et al., 2011).

Antimicrobial activity of marine algae could be attributed to combined effects of the high percentage of phenolic content, due to the presence of various phytochemicals (Govindasamy et al., 2011) and the presence of halogenated monoterpenes which are regularly present in marine algae (Cabrita et al., 2010). The type and amount of halogens present in the algal molecule also have a role in the overall defense against pathogenic Gram-positive and Gram-negative bacteria (Andrianasolo et al., 2006). The fats and fatty acids from marine algae also play an important role in the formation of many other bioactive secondary metabolites since some fatty acids have been shown to possess antibacterial activities (Barbosa et al., 2007; Oh et al., 2008).

The DCM extract of *P. rigidum* reconstituted in chloroform demonstrated antimicrobial activity against *E. faecalis, E. coli* and *S. epidermidis*. The ZOI for the extracts are 7. 41 ± 0.2, 7.42 ± 0 .31 and 6.21 ± 0.08 mm, respectively. Although these extracts were not very active *in vitro* compared to 10 µg/ml of ampicillin that showed activity in the range of 16.84 ± 0.22 mm (*E. coli*) and 8.94 ± 0.44 mm (*E. epidermidis*), they offer potential leads in the search for alternative antibiotics that could be active against *E. faecalis* and *S*. *sonnei.*

The DCM extract of *P. rigidum* reconstituted in ethanol showed a noteworthy result. 10 µg/ml of this extract showed trace antimicrobial activity of 6.18 ± 0.56 mm against *S. epidermidis*, while standard ampicillin of the same concentration showed no activity at all against *S. epidermidis*. The DCM extract of *P. cornutum* reconstituted in chloroform showed ZOI of 6.26 ± 007 mm against *S. epidermidis* while standard ampicillin showed no inhibitory activity against *S. epidermidis*. Concentrations higher than 10 µg/ml of extracts showed varying degrees of inhibition against all pathogens tested.

In addition, DCM extracts of *P. rigidum* and *P. cornutum* reconstituted in chloroform showed antimicrobial activity against both Gram positive (*E. faecalis* and *S. epidermidis*) and Gram negative (*E. coli* and *S. sonnei*) pathogens, which is an indication of a broad spectrum of activity.

The DCM extract of *P. rigidum* reconstituted in chloroform, was the most effective marine algae extract against the various pathogens. The ZOI and MIC obtained in this research are comparable to the results presented in similar work reported (Mostafa et al., 2017).

Conclusion

In this study presenting the first report of antimicrobial activity of Namibian marine alage, chloroform was the most suitable extraction solvent for antimicrobial compound extraction among the solvents used. It can be inferred from this study that the antimicrobial potential of *P. cornutum* and *P. rigidum* depend on the solvent medium used for extraction and the type of organism tested. *P. cornutum* and *P. rigidum* collected along the coastline of Namibia can be used as agents for the development of new drug leads for bacterial infections.

DCM extracts of *P. rigidum* (in ethanol) and *P. Cornutum* (in chloroform) are evidently potent antibiotics lead candidates *in vitro* against *L. monocytogenes* and *S. epidermidis*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This study was partly funded by the Namibian Commission for Research Science and Technology (NCRST) and the University of Namibia Staff Development Fund.

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Vol. 12(10), pp. 130-135, 15 March, 2018
DOI: 10.5897/AJPP2017. 4874
Article Number: 4EC3AD856268
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

A Study on Spurious and Not of Standard Quality Drugs in the State of Andhra Pradesh

Praveen Kumar G.* and Ravi Shankar A.

Drugs Control Administration, Government of Andhra Pradesh, Chuttugunta, Guntur-522 004, Andhra Pradesh, India.

Received 27 November, 2017; Accepted 6 February, 2018

Indian Pharmaceutical Industry is volume wise, the third largest in the world in terms of value and business of about USD 30 billion annually. Availability of quality medical products, especially drugs is important from the perspective of health of the consumers. The manufacture and sale of drugs in India is a licensed activity under Drugs and Cosmetics Act, 1940 and the Drugs and Cosmetics Rules, 1945. The use of ineffective, poor quality, harmful medicines can result in therapeutic failure, exacerbation of disease, resistance to medicines and sometimes death. Proper and effective enforcement of the Act is mandatory for curbing the sale, distribution and consumption of spurious which is not of standard quality medicines in any country, there by safeguarding the public health. In this paper, we report a post market quality survey of 3925 drug samples over a period of 30 months. Drugs Control Laboratory, Vijayawada, India during the said period (Jan, 2015 to June, 2017) 3925 samples was analyzed. It was found that, in the year 2015, 2016 and 2017 (up to June) the percentage of NSQ drugs was 5.70, 1.92 and 2.27%, respectively. The effective enforcement of Drugs and Cosmetics Act, 1940 is necessary at the field level to ensure that, safe medicines are made available to general public by and large.

Key words: Public health, spurious drug, not of standard quality drug, D and C Act 1940, Andhra Pradesh.

INTRODUCTION

The Indian Pharmaceutical Industry is presently the third largest in the world (volume wise). In terms of value, the size of the industry is approximately Rs. 2,00,000 Crores (USD 30 billion), out of which more than half is exported to different countries across the globe. The Indian Pharmaceutical sector has shown an exponential growth over the last 10 years. The growth of the sector over a short period of time has thrown up challenges for, up scaling the regulatory structure in the country. Availability of quality medical products, especially drugs is important from the perspective of health of the consumers. NSQ drugs may lead to not only sub-therapeutic dosage, but also give rise to anti-microbial resistance which is a major health concern.

With such a large domestic pharmaceutical market, circulation of Spurious and Not of Standard Quality Drugs (NSQ) can lead to grave and adverse consequences for both consumers and Drug manufacturers. Spurious and NSQ Drugs is a patient safety issue as it can challenge quality of treatment, lead to emergence of drug

*Corresponding author. E-mail: writetopraveenkumar@yahoo.com.

Table 1. Number of samples received and analyzed by DCL during 2015, 2016 and 2017 (up to June).

S/N	Month and Year	Number of samples received and analyzed
1	Jan, 2015 to Dec, 2015	1524
2	Jan, 2016 to Dec, 2016	1483
3	Jan, 2017 to June, 2017	918

resistance,spread of diseases and cause economic burden on the society. It is a shared responsibility of all stakeholders to tackle the challenges of Spurious and NSQ Drugs.

The manufacture and sale of drugs in India is a licensed activity under Drugs and Cosmetics Act, 1940 and Drugs and Cosmetics Rules, 1945. The licensees are required to comply with the provisions of the Act, Rules and the condition of the license granted to them by the licensing authorities for manufacture and sale of drugs. Drugs and Cosmetics Act, 1940 have elaborate provisions to check the production of spurious and substandard drugs in the country. The Act provides elaborate definitions of the terms spurious, adulterated and misbranded drugs for the purpose of taking penal actions against the offenders.

The use of ineffective, poor quality, harmful medicines can result in therapeutic failure, exacerbation of disease, resistance to medicines and sometimes death. It also undermines confidence in health systems, health professionals, pharmaceutical manufacturers and distributors. Medicine are lifesaving entities and thus more essential for the treatment which account for 20 to 60% of care cost while 50 to 90% of this cost is being paid by the patient, particularly in low and middle income countries (WHO, 2004). India is a developing country where more than 40% of the population survives on less than US $1 a day (Bate et al., 2011) and if a patient needs medicines he has to pay more than half of this.

In India, as per Drug and Cosmetic (D and C) act, 1940, under section 17, 17A and 17B poor quality drug comprises of misbranded, spurious and adulterated drugs, respectively (Government of India, 2005).

This article briefly present a glimpse of spurious and not of standard quality drugs in the state of Andhra Pradesh, India over a period of 30 months (from January, 2015 to June, 2017).

MATERIALS AND METHODS

Collection of samples

Under section 22.1.b (i) and (ii) of the Drugs and Cosmetics Act, 1940 (D and C Act) of India, about 3925 drug samples falling into 23 therapeutic categories were lifted and sent by 59 Drugs Inspectors of Andhra Pradesh state received in Drugs Control Laboratory (DCL), Vijayawada, India.

Period of sampling

The period of sampling in this context is January, 2015 to June, 2017.

Analysis of drugs and cosmetics

The samples received in DCL were assigned a unique number and analyzed as per the established monographs in standard pharmacopoeias like IP, BP, USP etc. In case of non-pharmacopeial patent and proprietary formulations, Standard test procedures were adopted for the analysis of such samples.

Reporting of analysis result

After the completion of the analysis of drug samples, the report was generated in Form-13 as prescribed in D and C Act, 1940 and opinion was declared as Standard Quality (SQ) or not of Standard Quality (NSQ) sample by the Government Analyst as per the powers delegated to him under section 20 of the D and C Act, 1940 and Rules under, 1945.

RESULTS

Received samples

During the sampling period from January, 2015 to June, 2017 drugs and cosmetics samples in 3925 numbers were analyzed in DCL, Vijayawada belonging to 23 different therapeutic categories as part of enforcement of Drugs and Cosmetics Act in the state of Andhra Pradesh in post market survey (Table 1).

Among the 3925 samples, highest number of samples was from anti-microbial group (1193 samples) which included anti-bacterials, anti-fungals, anti-virals, anti-helminths and anti-protozoals etc, following highest number of samples received belonged to the therapeutic category of analgesics which is 656 in number. Drugs acting on gastro-intestinal and respiratory tract were also high in number with 414 and 343 samples, respectively. Lowest samples belonged to Antivirals class which was only 07 for a period of 30 months.

Analysis of received samples

During the period of 30 months the analysis of received samples revealed that in the year 2015, out of 1524 samples analyzed 1437 samples were found to be of

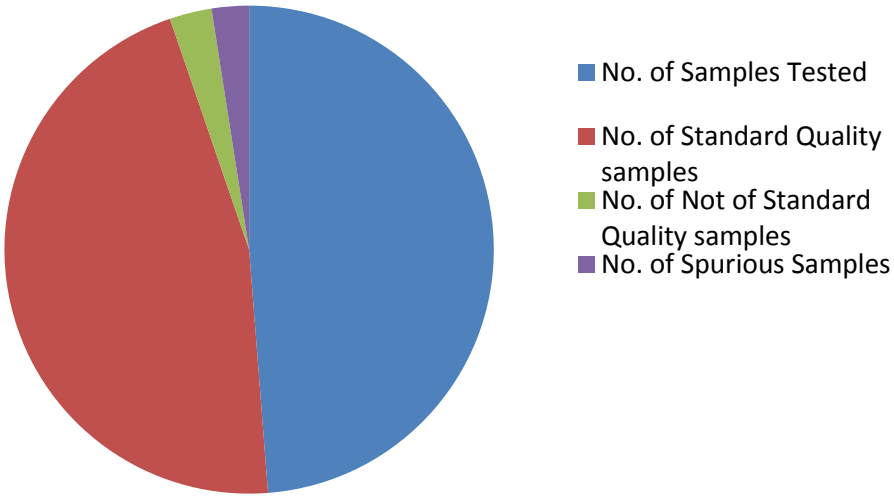

Figure 1. Number of samples analyzed was found to be of standard and not of standard quality in the year 2015.

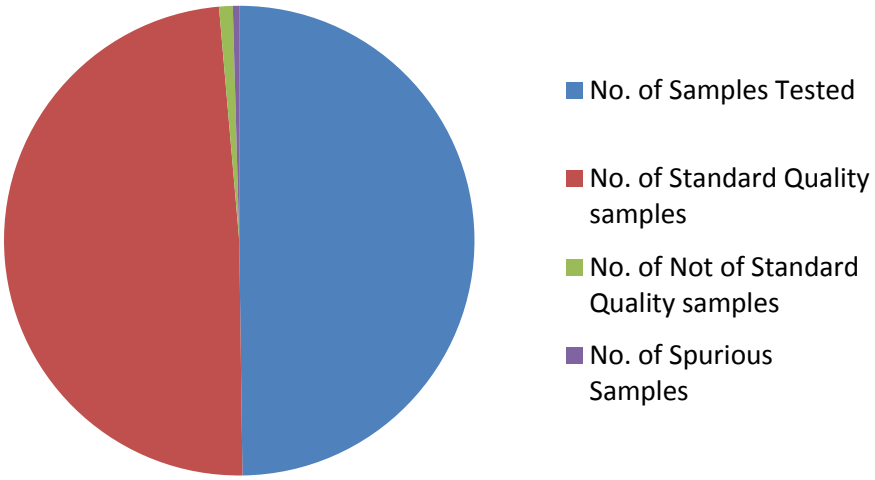

Figure 2. Number of samples analyzed was found to be of standard and not of standard quality in the year 2016.

standard quality while 87 samples were not. Also, out of 87 NSQs reported, 77 samples were demarcated as 'Spurious' due to the absence of active pharmaceutical ingredient. The percentage of NSQs in the year 2015 was 5.70% (Figure 1).

Totally, 1483 samples were analyzed in 2016 out of which 1455 were SQ samples and 28 were NSQ samples. Among 28 NSQs, 13 were identified as 'Spurious'. In the calendar year 2016 the percentage of NSQs was 1.92% (Figure 2).

Similarly, in the year 2017 from January to June, about 925 samples were received and analyzed and 904 samples were marked as SQ samples while 21 were found to be NSQ samples. No spurious samples were identified in the same year and the percentage of NSQs was found to be 2.27% (Figure 3).

Test parameters Vs NSQs

It was found that, of the total NSQs in the year 2015 and 2016, highest NSQs were with reference to failing in Identification test for active ingredients which were 77 and 14 in number, respectively. In these two years, samples which failed in dissolution test were the lowest with only two samples each year (Table 2).

In the year 2017, interestingly highest number of samples failed in dissolution test is 13 and lowest number

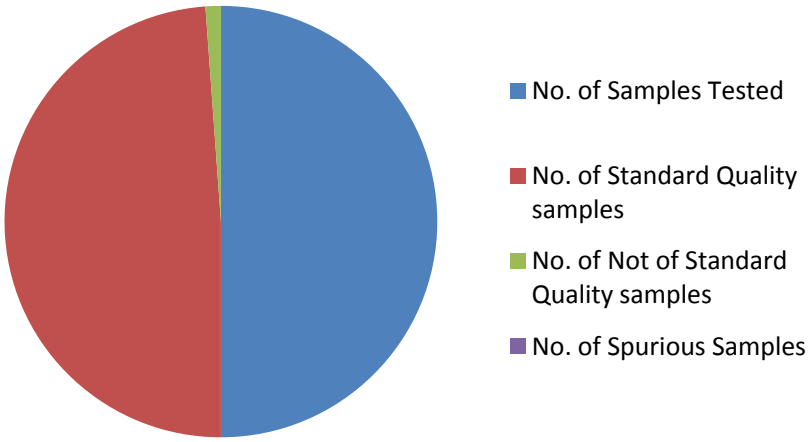

Figure 3. Number of samples analyzed was found to be of standard and not of standard quality in the year 2017.

of samples failed in uniformity of weight was only 2 in a span of six months (Table 3).

DISCUSSION

Poor quality drug or substandard product encounters a major stringent issue for the global health system (Bate et al., 2011) which cannot be ignored. In most streamlined regions of the globe like Japan, Canada, Australia, New Zealand, the United States of America and most of the European Union, hardly 1% of the market value products are counterfeit, developing countries like Africa, Latin America and many parts of Asia may markedly be the seller and producer of spurious/falsely-labeled/falsified/counterfeit (SFFC) drugs medicines (WHO Report, 2012).

In the current study, it was found that over a period of 30 months the percentage of NSQs ranged between 1.92 to 5.70%. In the year 2015 out of 1524 samples analyzed, 1437 samples were declared as of standard quality and 87 samples were not. The reason for such high percentage of NSQs in the year 2015 could be due to declaring 77 samples as NSQs due to their spurious nature (Figure 1), wherein active pharmaceutical ingredient was completely absent.

In the year 2016, the number of NSQs declared was restricted to only 28, of which 14 were found to be spurious, which failed to meet identification test of labeled drug or had zero active ingredient. The overall percentage of spurious drugs was about 2.3% for a period of 30 months (Figures 1 and 2). The high percentage of encountering spurious drugs in the years 2015 and 2016, belonged to only one manufacturer namely, M/s Avya Health Care, with its fake manufacturing unit in Himachal Pradesh state. The spurious drug samples declared as NSQ belonged to therapeutic categories which consists of Antibiotics, Analgesics, Anti-emetics, Anti-cough and cold, Anti-protozoal, Iron and Vitamin and Anti-ulcers in the form of tablets, capsules and syrups/suspensions. Substandard drugs receive a lot less attention than spurious drugs. According to official data, substandard drugs outnumber spurious drugs amongst poor quality drugs (Maulik et al., 2015).

In the present study it was found that, 88.5% of samples in the year 2015 were NSQs with respect to Identification test (spurious samples) and the other tests accounted for 11.5% of NSQs. Majority of the samples tested in the year 2015 were NSQs with respect to one category of drug i.e. anti-microbials and hence spurious. The spurious nature of antibiotics could result in increased anti-microbial resistance, treatment failure and side effects (Okeke et al., 1999; Syhakhang et al., 2004) whereas, in the year 2016 spurious samples accounted for 46.4% of total NSQs. In contrary, in the year 2017, highest number of NSQs was declared with respect to dissolution test which accounted for about 61.9% of total 26 NSQs and next were in assay for active ingredients with 38.09%. A similar trend of NSQs was reported with highest number of samples failing in dissolution and assay in a recent world's largest drugs quality survey conducted by National Institute of Biologicals, India (NIB Drug Survey, 2014 to 2016). The reason for such higher number of samples failing in dissolution test could be due to the insoluble nature of binding matrix or changes in formulation constitution etc which does not permit to release the drug from dosage form in stipulated time and thereby poor pharmacological action. Another reason for failure in dissolution test of tablets could be due to storage conditions particularly in tropical country like India (Johnston and Holt, 2013).

In a nutshell, it is estimated that, the post-market survey (for a period of 30 months) under the Drugs and

Table 2. Therapeutic category wise number of samples received and analyzed in DCL, Vijayawada declared as NSQs for a period of 30 months.

S/N	Therapeutic category	Number of Samples received/ Year			Total number of samples received	Number of NSQs		
		2015	2016	2017 (up to June)		2015	2016	2017 (up to June)
1	Anaesthetics	4	8	2	14	-	3	-
2	Analgesics	228	289	139	656	14	2	1
3	Anti-cancerous	3	1	3	7	-	-	-
4	Anti-depressants	11	16	8	35	-	-	-
5	Anti-diabetics	67	63	50	180	-	-	1
6	Anti-emetics	12	15	6	33	-	-	1
8	Anti-epileptics	9	12	14	35	2	-	2
9	Anti-histamines	82	83	33	198	-	3	1
10	Anti-hypertensives	40	77	32	149	2	-	1
11	Anti-lipidemics	14	5	11	30	1	-	-
12	Anti-microbials	476	430	287	1193	54	9	12
13	Anti-psychotics	11	16	12	39	-	-	-
14	Anti-pyretics	63	23	28	114	2	4	1
15	Dermal related	9	5	1	15	-	-	-
16	Diuretics	10	15	8	33	-	1	-
17	Electrolytes	3	6	0	9	-	-	-
18	Enzymes	9	3	4	16	-	-	-
19	Gastrointestinal related	145	176	93	414	8	7	4
20	Hormones	9	9	4	22	-	-	-
21	Anti-helmintics	5	4	0	9	-	-	-
22	Non-steroidal Anti-inflammatory drugs	22	7	0	29	-	-	-
23	Others	21	16	26	63	-	-	-
24	Respiratory tract related	142	108	93	343	1	1	-
25	Steroids	39	26	28	93	-	-	-
26	Surgicals	8	11	7	26	-	3	-
27	Vitamins & Minerals	54	48	23	125	7	3	-
28	Cosmetics	28	11	6	45	-	-	2

Cosmetics Act, 1940 in the state of Andhra Pradesh revealed that, the percentage which is not of standard quality drugs in the state of Andhra Pradesh on an average, was 3.95%. This percentage is fairly higher than the national average of 3.16% during 2014 to 2016.

It is further understood that, manufacturers have to follow strict cGMP guidelines to see that there is a reduction in number of NSQs which can take

Table 3. Test Parameter wise number of Not-of Standard Quality samples declared over a period of 30 months.

S/N	Test Parameter	Number of NSQs		
		2015	2016	2017 (up to June)
1	Description	06	09	04
2	Identification	77	14	-
3	Uniformity of Weight	02	01	02
4	Disintegration	-	04	01
5	Dissolution	02	02	14
6	Assay	04	06	10

care of the supply chain management for better receipt of drugs to the consumers in a form it intended to be. Government drugs procurement agencies must also check the medicines procured by them for meeting the standards. Drug regulation through stringent law enforcement in state and central regulators is also an effective step to curb the movement of spurious and sub-standard drugs in the market.

Conclusion

In the absence of effective medicines regulation, increased globalization of the pharmaceutical trade can lead to the proliferation of harmful, ineffective, substandard and counterfeit medicines on national and international markets. With the current study it is evident that, the movement and market of spurious and NSQ drugs is near to national average in the state of Andhra Pradesh.

Substandard/counterfeit antimicrobial drugs represent an expanding problem throughout developing countries with considerable consequences for global public health. Well-designed studies are needed to determine the magnitude of this problem. A substandard concentration of the active ingredient was the main reason for low quality, which can lead to increased morbidity and mortality and emergence of antimicrobial resistance.Further, in order to curb the movement of such sub-standard medicines further effective enforcement is required at the field level to ensure that, safe medicines are made available to general public by and large in the interest of the state and nation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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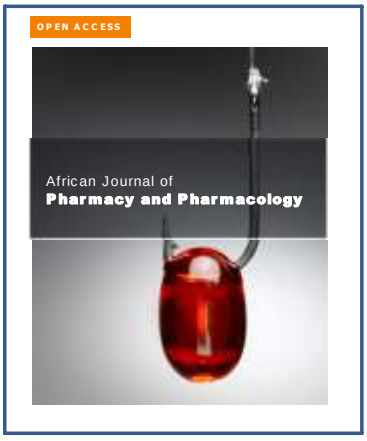

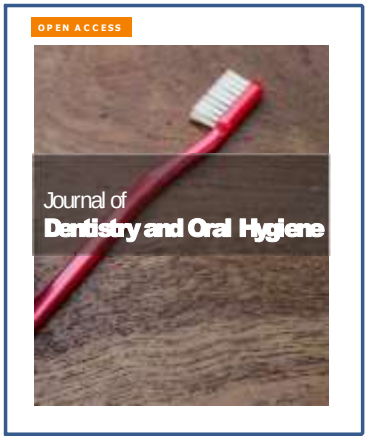

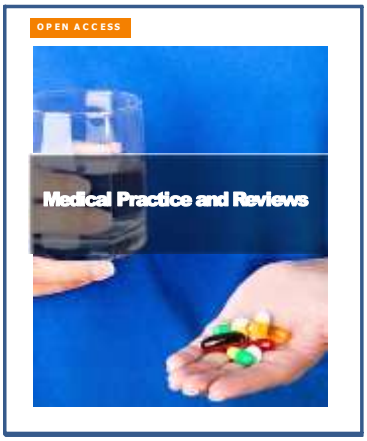

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