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Full Length Research Paper

A quality control study of ibuprofen tablets available in the formal and informal market in Harare, Zimbabwe

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This research provided quantitative data for the quality of randomly selected ibuprofen tablets that are sold in both formal and informal market in Harare, Zimbabwe. Fourteen batches of ibuprofen tablets comprising of sixty tablets per batch were randomly selected from retail pharmacies and informal traders over a period of six months. Quality control tests which included physical appearance, uniformity of mass, disintegration test, friability, *in vitro* dissolution test, spectrophotometric and High Performance Liquid Chromatography (HPLC) profiling of the samples were carried out. Three out of the fourteen batches failed the uniformity of mass test. Those that failed were from the informal market. One out of these also failed the quantitative chemical assay and dissolution test. Four other ibuprofen batches were according to labelling from the same manufacturer but had differences in the packaging used therefore raising suspicion of the possibility of counterfeiting. Two more ibuprofen samples were from the same manufacturer but the appearance of these samples differed in shape size and colour and therefore failed the appearance test. The other batches passed all the tests done. All the samples tested contained the stated active pharmaceutical ingredient (API), ibuprofen. However, the other three samples were reported as substandard as they failed to meet the WHO set standards for either uniformity of weight, chemical assay test or dissolution test.

Key words: Ibuprofen, quality counterfeit, active pharmaceutical ingredient (API), friability, high performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FTIR).

INTRODUCTION

Ibuprofen is an over-the-counter antipyretic, analgesic and non-steroidal anti-inflammatory drug that is widely used for the relief of pain and inflammation caused by headache, toothache, back pains, menstrual pains, minor injuries and arthritis. The ability of ibuprofen to treat a wide case of mild to serious pain makes it a popular medicine. With many new medicinal products released into the market regularly, it is increasingly difficult to keep

track of the safety of every product and this failure to keep track of medicinal products has led to an influx of counterfeit or substandard products (Yankus, 2006). Fake drugs have capacity to deceive, particularly if they are copied to make it look like the original product so that purchasers are unlikely to be suspicious (Nsimba, 2008). Analytical methods and anti-counterfeit technologies have therefore been developed to determine if products

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in the market are of good quality and reduce the risk substandard products may pose to consumers (Arzamastev et al., 2004; Deisingh, 2005; Bansal et al., 2013; Shah et al., 2010). Reports suggest that in a number of developing countries, there is a high incidence of the availability of substandard drugs mainly due to poor monitoring programs (Morris and Stevens, 2006; WHO, 2006; Kelesidis et al., 2007).

According to WHO (2006), poorly manufactured pharmaceutical products may be classified as either substandard or counterfeit. In the same report, the WHO describes substandard products as those that contain the active pharmaceutical ingredient (API) but do not conform to the quality requirements for that product such as incorrect quantities of the API or fails other physical tests such as the weight variation test, friability, hardness, disintegration test, thickness and diameter test. The WHO standards further describe a counterfeit drug as one that is deliberately and fraudulently mislabeled with respect to identity and or source.

In this study, the quality of ibuprofen tablets found in the formal and informal market in the city of Harare, Zimbabwe was reported. The chemical structure of ibuprofen is shown in Figure 1.

MATERIALS AND METHODS

Sampling

Fourteen batches of ibuprofen tablets comprising of sixty tablets per batch were sampled over a period of six months in Harare, Zimbabwe's largest city. The sampled tablets from the formal and informal market originated from Kenya, India, Cyprus and China. Table 1 summarises the samples obtained.

Chemicals and chemical reagents

Chemicals used for various experiments were of analytical reagent grade unless otherwise stated. Ibuprofen reference standard was obtained from United States Pharmacopoeia (Rockville, USA), Lot Numbers KOJ009. Potassium dihydrogen orthophosphate and sodium hydroxide were purchased from Surechem Products Ltd., RSA and orthophosphoric acid from Minema Ltd., RSA. HPLC grade methanol was purchased from Merck (Pvt) Ltd. Water for HPLC analysis was Millipore purified.

Equipment

A Shimadzu LC-20AD HPLC equipped with a SPD-2AD UV/Vis detector was used for HPLC profiling and quantitative determination of API. A Distek apparatus (USA) was used for dissolution tests and a Shimadzu UV1800 UV/Vis spectrophotometer was used for quantitative determination of API dissolved in the media. For the friability test, an Erweka friabilator (Germany) was used.

Sample characterization methods

Test for uniformity of mass

The method for uniformity of mass determination was adapted from

Okunlola et al. (2009). Exactly 20 tablets were weighed individually and relative standard deviations were used to determine the variation in mass.

Friability test

The friability test method was adapted from Ngwaluka et al. (2009). Exactly 20 pills were weighed initially and then placed in a friabilator for 4 min. The final mass was then determined. The friability of the tablets was calculated using equation 1:

$$F (\%) = \frac{w_1 - w_2}{w_1} \times 100 \quad (1)$$

Where F % is the percentage friability, w_1 is the initial weight and w_2 is the final weight. The friability value of tablets should be less than 1.0%.

Disintegration test

Disintegration test method was adapted from Dewan et al. (2013). Exactly six pills were individually suspended in 800 mL of water, equilibrated to and maintained at $37 \pm 0.5^\circ\text{C}$. The disintegrator operated for 30 min. The time taken for each tablet or capsule to completely disintegrate was recorded.

API determination

An HPLC method adapted from Sunaric et al. (2013) Eraga et al. 2015 was used for the determination of API in the ibuprofen samples. Exactly 20 pills were weighed and ground into a fine powder. A quantity of the powdered tablets containing 200 mg of ibuprofen was weighed into a 100 mL volumetric flask, shaken with about 30 mL of the mobile phase and sonicated for 30 min. The mixture was allowed to cool to room temperature and made up to volume with the mobile phase. This mixture was then centrifuged for 5 min at 2500 rpm. The supernatant liquid was used as the sample solution. The chromatographic conditions were as follows: Mobile phase (methanol: water: orthophosphoric acid (750:247:3), C18 5 μm Luna column: 4.6 mm x 15 cm, flow rate: 1.0 mL/min, injection volume: 10 μL , detection: 264 nm. The percentage released API was calculated using Equation 2.

$$\text{Content} (\%) = \frac{A_u \times C_{std} \times \text{potency} \times \text{sample dilution factor}}{A_s \times \text{specific gravity} \times \text{label claim}} \times 100\% \quad (2)$$

Where, A_u is the mean area response of the sample and A_s is the mean area response of the standard, C_{std} is the concentration of standard in $\mu\text{g/mL}$.

Dissolution test

The dissolution test method was adapted from Kulkarni et al. (2011) and Giri et al. (2013). Exactly 6 tablets per batch were suspended in 900 mL of phosphate buffer solution of pH 7.2 that had been brought to an equilibrium temperature of $37 \pm 0.5^\circ\text{C}$. The dissolution process was monitored over a 90 min period. Aliquots of 10 mL were withdrawn at intervals of 5, 10, 15, 20, 30, 45, 60 and 90 min and filtered through a 0.45 μm filter. 1 mL of the filtrate was pipetted into a 20 mL volumetric flask and made up to volume with the dissolution media. The concentration of API was determined using a UV/Vis spectrophotometer set at 220 nm. The percentage API released was calculated using Equation 3.

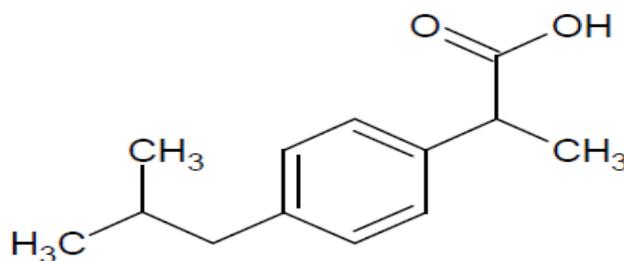


Figure 1. Chemical structure of ibuprofen.

Table 1. Samples analysed.

Sample name	Date of manufacture	Expiry date	Labelled strength (mg)	Market
A	10/2013	09/2017	200	Informal
B	01/2015	12/2016	200	Informal
C	10/2013	10/2018	200	Formal
D	Not given	09/2017	200	Informal
E	11/2013	10/2017	200	Informal
F	05/2015	04/2017	200	Informal
G	07/2015	07/2020	200	Formal
H	Not given	04/2019	200	Informal
I	01/2014	12/2016	200	Formal
J	Not given	02/2017	400	Formal
K	Not Given	11/2016	200	Formal
L	01/2015	12/2017	400	Formal
M	05/2015	04/2017	200	Informal
N	05/2015	04/2017	200	Informal

$$\text{Content (\%)} = \frac{A_u \times C_{\text{std}} \times \text{potency} \times \text{sample dilution factor}}{A_s \times \text{specific gravity} \times \text{label claim}} \times 100\% \quad (3)$$

Where, A_u is the mean area response of the sample and A_s is the mean area response of the standard, C_{std} is the concentration of standard in $\mu\text{g/mL}$.

RESULTS AND DISCUSSION

Uniformity of mass

The results for uniformity of mass for selected batches from the informal market are illustrated in Figure 2. From the figure, it can be observed that three batches failed the uniformity of mass test as stated in the British Pharmacopeia (2015). The batches consisted of two from the same manufacturer and were all from the informal market.

It can also be observed that seven out of twenty tablets of batch D failed the first condition of not more than $\pm 5\%$ whilst one of the tablets had a deviation more than $\pm 10\%$. Three tablets of the tested batch H had variation of more than $\pm 5\%$ whilst one had more than $\pm 10\%$. Avbunudiogba et al. (2013) suggested that variations in the uniformity of mass within the same batch is a strong

indication of poor manufacturing practises that may lead to different dosages on different occasions if the mass of the API differ from tablet to tablet. Batches D and H were from the same manufacturer.

Karmakar and Kibria (2012) described further that high variation in weight may be an indication of corresponding variation in the drug content. Accordingly, higher percentage variations in tablet weight may mean the active pharmaceutical ingredient will differ from one tablet to the next which may be detrimental to a patient who may end up being under dosed and overdosed on different occasions. However, it is also possible that since these ibuprofen tablets are film coated, poor manufacturing practices during the coating process may lead to high inconsistency in the amount of coating applied per tablet. This may lead to large variations in the weight but not necessarily affecting the amount of API available per tablet but probably affect the rate at which the API is released. Eleven out of the tested fourteen batches passed the uniformity of weight test.

Friability

Teklu et al. (2014) reported that friability tests may

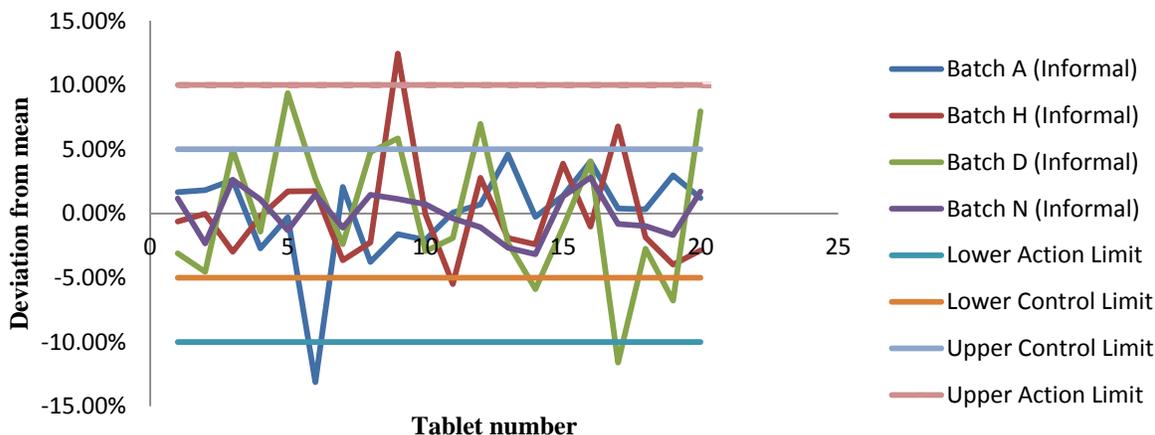


Figure 2. Uniformity of mass results for selected ibuprofen tablets batches.

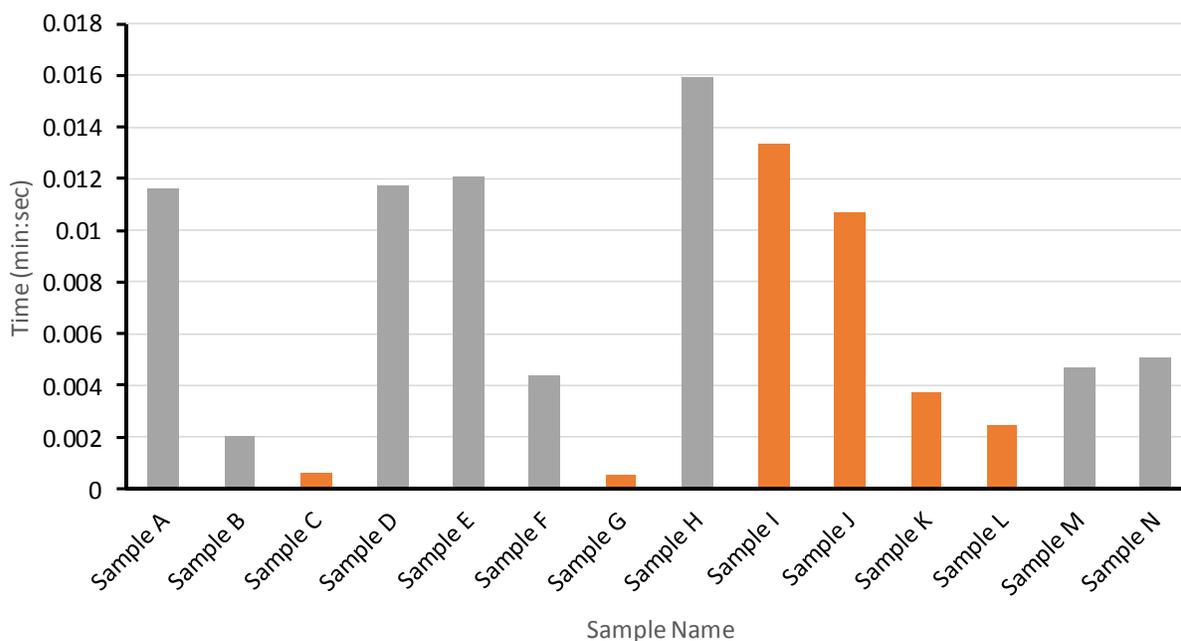


Figure 3. Disintegration time results for ibuprofen tablets from fourteen batches.

indicate the resistance of the tablets to external pressure from manufacturing, storage, handling during shipment and transportation. All batches tested had % friability of less than 0.1%. The results show that all samples passed the friability test set by the WHO (2014). This may be attributed to the film coating that protects the tablet against abrasion.

Disintegration time results

The British Pharmacopoeia (2015) sets limits for

disintegration of coated tablets as 30 min. All fourteen batches passed the disintegration test although some samples from certain batches had relatively high disintegration times ranging from 15 min 26 s to 22 min 56 s (Figure 3). Notably, samples from batches A, D, E, H, I and J had disintegration times ranging from 15 min 26 s to 22 min 56 s. Samples from batches A, D, E and H were from the informal market while those from batches I and J were from the formal market.

According to a report by Cardot et al. (2007), disintegration is the break down process of tablet into smaller particles and is the first step towards dissolution.

Table 2. Results for HPLC analyses.

Sample name	Replicate 1	Replicate 2	Replicate 3	Average	RSD
A	105.47%	104.62%	103.91%	104.66%	0.74%
B	100.33%	101.03%	99.57%	100.31%	0.73%
C	101.68%	101.89%	101.45%	101.67%	0.22%
D	104.86%	103.77%	103.21%	103.95%	0.81%
E	99.97%	98.23%	99.71%	99.30%	0.95%
F	96.63%	96.05%	97.11%	96.60%	0.55%
G	100.71%	102.39%	101.20%	101.43%	0.85%
H	123.67%	121.63%	123.22%	122.84%	0.87%
I	100.65%	100.67%	101.76%	101.03%	0.63%
J	101.06%	99.10%	100.12%	100.09%	0.98%
K	97.27%	96.69%	96.43%	96.80%	0.45%
L	98.93%	100.34%	99.23%	99.50%	0.75%
M	96.90%	96.24%	97.24%	96.79%	0.53%
N	95.28%	96.09%	97.23%	96.20%	1.02%

It can be suggested that the longer the disintegration time, the longer the time taken before the API is absorbed by the body. It may therefore be assumed that the longer the disintegration time, the longer the time taken before the API is dissolved and absorbed by the body (Esimone et al., 2008; Niharika et al. 2013).

It was observed that batches A and E were from the same manufacturer, batches D and H from another manufacturer and so were batches I and J. From these observations, it could be concluded that the relatively high disintegration times observed could be related to the different manufacturing processes. Excipients such as binders and type of coating used play a major role in the rate at which a tablet breaks down into smaller particles and the way the API is released. If the binders have affinity for the API then more time is required before the API is released for absorption.

HPLC analytical results for API

The results for HPLC analysis are shown in Table 2. It can be concluded that batches except ibuprofen sample H met the standard requirements. Samples from batch H contained $122.84 \pm 0.87\%$ of ibuprofen. It can be suggested that there is a high possibility that any patient who uses this sample will be overdosed by more than 12% of the maximum allowed ibuprofen per tablet. This sample happened to be from the informal market.

Dissolution test results

Dissolution of an oral pharmaceutical product is important because it gives an indication of the percentage of the drug that is available for absorption after a specific time.

It therefore follows that high dissolution rate means high bioavailability of the drug content. The results for dissolution profiles are illustrated in Figure 4. All batches except batch H passed the dissolution test as more than 85% of the API was released within the stipulated time of 60 min. Although, the average content of API released for sample H is 97.38%, the % RSD of 13.58 means some tablets within the sample had a % API dissolved of below (Q) = 85%. The results show that samples from batch H were substandard as they failed the dissolution test.

From the dissolution profiles in Figure 4, samples from batches C, I and L had the highest dissolution efficiencies at T_{15} . These three products were obtained from retail pharmacies. Samples from batches A, B and D only had dissolution efficiencies comparable to those of samples from batches C, I and L after 30 min and these happened to be from the informal market.

The above results may show the risks of purchasing a pharmaceutical product from an informal market. Since most of the people who purchase these products use them for the relief of pain and inflammation, the longer it takes for the drug to be bioavailable, the higher the chances of an overdose. A different drug may be ingested to relieve pain when the other one is still in the system leading to improper drug interactions.

A pharmaceutical product of oral dosage form normally contains a drug substance known as the active pharmaceutical ingredient (API) and its excipients. The proportion between the excipients, the type of excipients (grade) and the manufacturing process may affect the bulk properties and absorption properties of the product. This gives each product a general dissolution pattern as illustrated in the Figure 4. Although, the same API-ibuprofen was analysed, differences in the manufacturing process, type and proportions were used for different brands leading to different dissolution profiles with the

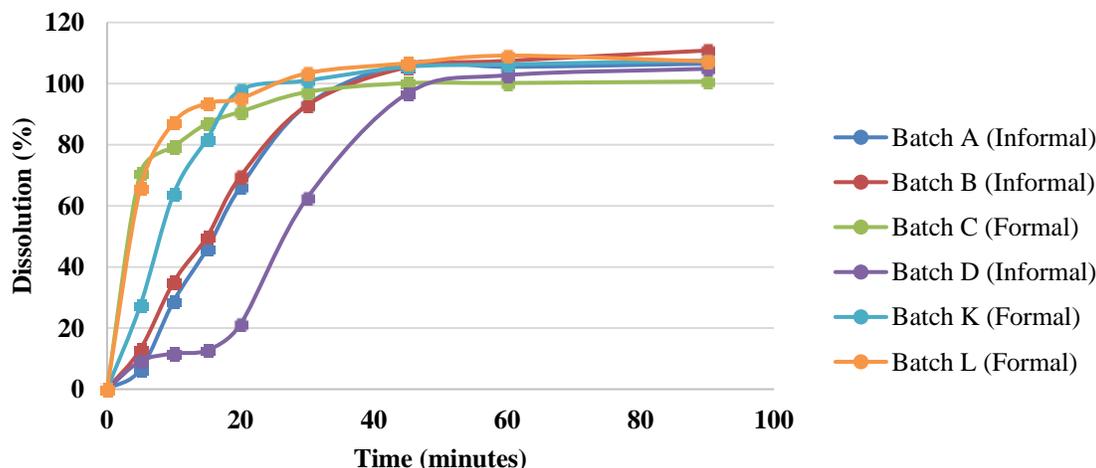


Figure 4. Dissolution profiles of different batches of ibuprofen tablets from selected batches.

efficiencies increasing in the order $D < A < B < K < I < L < C$ at T_5 . It can also be observed from the results that samples of batches of the retail pharmacies generally had dissolution efficiencies consistent with regulatory requirements as compared to those in the informal market.

Batch to batch consistency

If a manufacturer is using a validated manufacturing process, results from one batch to the next are expected to be comparable such as those illustrated in Figure 5a and c showing results of samples from a retail pharmacy. More than 85% of the API had been released in 15 min for both batches. Similar profiles were also observed (Figure 5d) for the samples from batches D and H from the same manufacturer. Although, very low dissolution efficiencies were noted at T_{15} , the dissolution profiles are similar in the rate at which the API is released.

The same however cannot be said for samples from batches B, F, M and N as illustrated in Figure 5b. Four different batches from the same manufacturer were analysed. Three of these batches (F, M and N) were manufactured in the same month according to the labels, and gave similar results but these three results differed from the results of the batch (Sample B) that had been produced earlier (Table 1).

From Figure 4, it can be noted that samples from batch D had the lowest dissolution efficiency at T_{15} . This strongly correlates with disintegration profiles in Figure 3 that shows batches D and H from the same manufacturer having relatively the highest disintegration time.

This was not the case with samples from batches B, F, M and N in Figure 5b. The results deviate from the strong correlation suggested by Esimone et al. (2008). Samples from batch B had the least disintegration time of the four

batches but it did not release the API as efficiently as the other three batches. This may raise concern to a regulator when presented with these results as samples from batch B had a different packaging as compared to the other three batches. The two packaging used shows variation in the way the batch number and date of expiry were printed. Samples from batch B had the batch details embossed on the blister pack whereas details for samples from batches F, M and N were stamped.

Visual appearances

There was also a difference in the font used to label the brand name with ibuprofen sample B having small letters throughout, whereas the other three batches had capital letters at the beginning and at the end of the product name, for example one sample being branded as 'sample-ab', whereas the other three samples were labeled as 'Sample-AB'. The dimensions of the blister packs used also differed with ibuprofen samples from batch B being 9.5 x 3.7 cm, while the other three batches had 8.8 x 3.7 cm. It is possible for a manufacturer to change packaging for the same product in the same year but it also has an effect of introducing room for counterfeiting as there are chances that it will look like the original. Differences of this nature gives room for counterfeiters as a fake product may be marketed so this sample was suspected to be a counterfeit where the branding may be falsified.

Ibuprofen samples from batches D and H also differed in appearance although they were manufactured under the same brand name. Shape, size and colour of the tablets differed as samples from batch H were smaller in diameter and more intense in colour. This raises suspicion of counterfeiting as samples from batch H failed the assay and dissolution tests that followed. Both

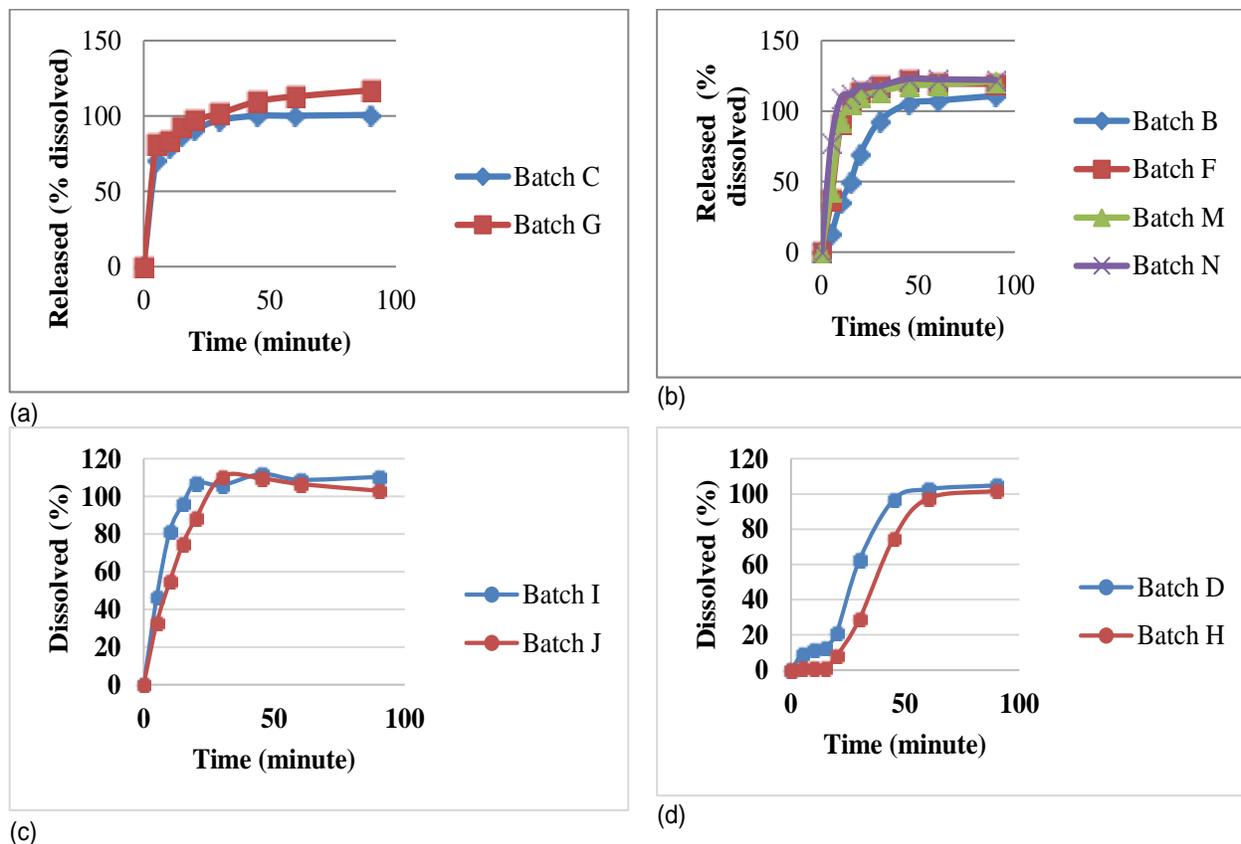


Figure 5. Batch to batch consistency of dissolution profiles.

samples were different batches from the informal market. All batches from both informal and formal market did not contain a leaflet or package insert to explain dosage, medicine content, adverse effects, medicine action and how the medicine should be taken. This observation has serious health effects as not all patients ask for this information before use of medication and there is no guarantee on how accurate the information may be disseminated from supplier to end user.

HPLC profiling

Figure 6a to e shows variation in the HPLC profiles. Only one peak appeared in the ibuprofen standard profiles (Figures 6a), whereas profiles for ibuprofen samples from batches A, B, E, F, K, M and N had more than a single peak. All these samples except samples from batch K were from the informal market. Samples from batches B, F, M and N were samples from the same manufacturer and samples from batches A and E were from another manufacturer. Although, samples from batch K was from a formal market, it was not sold in the original packaging and therefore did not contain the brand name of the product, date of manufacture and country of origin for the

product. The two peaks that were detected in these samples could have been excipients that are soluble in the mobile phase, impurities (either intentional such as undeclared APIs) or degradation products.

Conclusion

From the results obtained in this research, it can be concluded that out of the fourteen batches, three failed the uniformity of weight test and these were from the informal market. One out of these three that failed the uniformity of weight test also failed the quantitative chemical assay test and the dissolution test. Four other ibuprofen batches were from the same manufacturer but had differences in the packaging used therefore raising suspicion of the possibility of counterfeiting. Two more ibuprofen samples were from the same manufacturer but the appearance of these samples differed in shape, size and colour and therefore failed the appearance test. The other batches passed all the tests done. All the samples tested contained the stated API ibuprofen. However, the other three samples were reported as substandard as they failed to meet the WHO (2015) set standards for either uniformity of weight, chemical assay test or

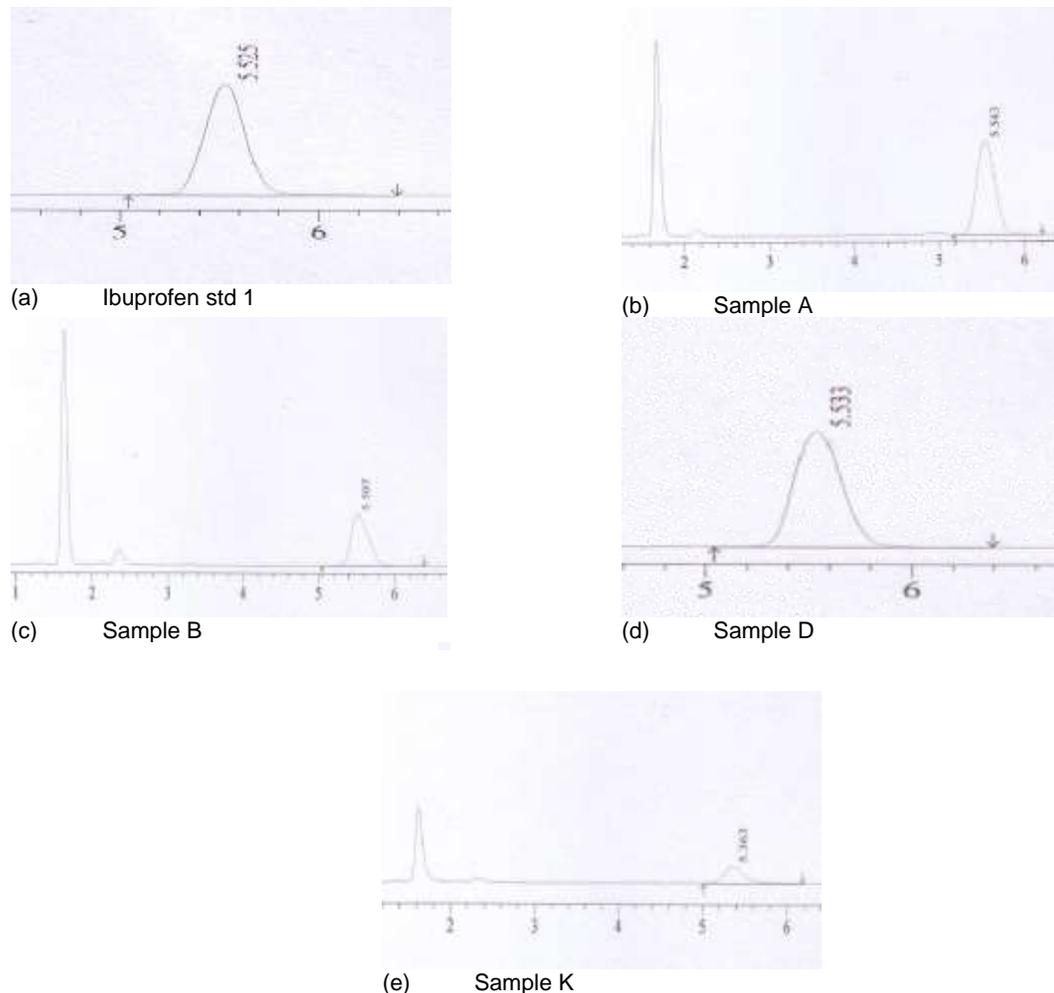


Figure 6. Ibuprofen HPLC profiles.

dissolution test.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antibacterial potential of the 80% methanol and chloroform extracts of *Clematis hirsuta*

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The leaves and roots of *Clematis hirsuta* Perr and Guill is traditionally applied to heal respiratory tract and for the treatment of various animal diseases in different regions of Ethiopia. The objective of this work is to evaluate the antibacterial activities of *C. hirsuta*. The leaves were collected and air dried in shade at room temperature, made into powder and was soaked in 80% methanol and chloroforms (1 g: 10 ml). The powder was placed in a shaker for 72 h at room temperature. The extract was prepared in 3% Tween 80 for antibacterial test. The antibacterial activities and the minimum inhibitory concentration (MIC) test were determined by paper disk diffusion and agar dilution methods respectively. The 80% methanol and chloroform extract of the leaves of *C. hirsuta* showed significantly higher inhibition zone than the negative control on some pathogenic bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella boydii* and *Salmonella thyphi*), but the extracts had significantly lower inhibition zone than the standard drugs (chloramphenicol and ampicilin). The chloroform crude extract of the leaves *C. hirsuta* showed the best inhibition zone (12.33±0.50) on *P. aeruginosa* at 200 mg/ml concentration. The chloroform extract of *C. hirsuta* had the lowest MIC and minimum bactericidal concentration (MBC) at 3.125 and 6.25 mg/ml on *P. aeruginosa*, respectively. The 80% methanol *C. hirsuta* leaf extract was not toxic at 500, 1000 and 2000 mg/kg body weight to albino mice.

Keywords: Inhibition zone, acute toxicity, minimum inhibitory concentration, minimum bactericidal concentration.

INTRODUCTION

Medicinal plants are used in spiritual therapies, manual techniques and applied to treat, diagnose or prevent illness (WHO, 2013). In Ethiopia, more than 80% of the people use traditional medicines to apply them in various types of infections and disorders (Bekele, 2007). It is reported about 200,000 natural products are known, but most of drugs are isolated from higher plants and microorganisms (Ramawat and Merillon, 2007). However, traditional practitioners in Ethiopia use the plants without

scientific dose optimization. It has reported that different parts of the plant are used traditionally to treat different types of infections (Ogbulie et al., 2007). *Clematis hirsuta* belongs to family Ranunculaceae. It is perennial woody climber and measures up to 1 to 4 m long. It bears oppositely arranged compound leaves. Flower is borne in panicles with white or yellow color. *Clematis* species are known to have secondary metabolites including glycoside ranunculin, saponins, tritreprenoid, alkaloids and

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cyanogenetic glycosides (Trease and Evans, 1996). Thus the *Clematis* species are used as medicinal plants. Mainly *C. hirsuta* are used in folk medicine worldwide especially in Asia as anti-inflammatory, analgesics and anti-rheumatics (Al-Taweel et al., 2007). The petroleum ether and butanol extracts of *C. hirsuta* revealed the presence of sterols and triterpenes and has anti-inflammatory on rat model (Abdel-Kader et al., 2008). In the other studies, the methanolic and ethanolic extracts of the related species (*C. ispahanica* and *C. orientalis*) shows antibacterial activity (Raei et al., 2013).

In Ethiopia, *C. hirsuta* with local name "Azo tero" is used in folk medicine as analgesics and anti-rheumatics. Roots of *C. hirsuta* and *Sida schimperiana* (Malvaceae) are crushed, powdered and mixed with water for oral and nasal administration to treat blackleg and *C. hirsuta* accounts 36.73% of all medicinal plant considered in the study for the treatment of various animal ailments in Bale Districts (Yineger et al., 2007). *C. hirsuta* is also used to heal respiratory tract problem and cataract in Meinit ethnic group, southern part of Ethiopia (Giday et al., 2009). Thus, the study aimed at evaluating the antibacterial activities of the 80% methanol and chloroform extracts of leaves of on *C. hirsuta* some pathogenic bacteria.

MATERIALS AND METHODS

Methanol (Reagent chemical Services Ltd., United Kingdom), chloroform (Merck KGaA, 64271, Darmstadt, Germany), nutrient agar (Oxoid LTD., Basingstoke, Hampshire, England), Muller-Hinton agar (Oxoid LTD., Basingstoke, Hampshire, England), barium chloride sulfuric acid (SDFCL Fine Chemical Ltd., Mumbai, India), Tween-80 (Uni-Chem Chemical Reagents), sodium chloride (Nike Chemical, India), ampicillin (Oxoid Ltd., United Kingdom), chloramphenicol (Oxoid Ltd., United Kingdom), barium chloride (BDH Chemicals Ltd. Poole, England) were used.

Plant collection and identification

The fresh leaves of *C. hirsuta* were collected from natural vegetation in Dejen district, Eastern Gojjam Zone, Amhara Regional State, Ethiopia in June 2014. The specimen were identified and authenticated by Mr. Melaku Wondafrash at National Herbarium, Department of Biology, Addis Ababa University. The voucher specimens were deposited in the herbarium and named with *C. hirsuta* (ah004).

Preparation of solvent extraction

The fresh leaves of *C. hirsuta* were washed three times with tap water and once with sterile distilled water. After wash, the plant materials were air dried in shade at room temperature (25 to 30°C) for two weeks until it became completely dry. Following the drying process, about 0.3 kg leaves was powdered and sieved through a fine mesh (Canadian Series sieves with 5×10^{-4} opening) and stored in dry bottle at temperature for further use as described in Subbarayan et al. (2010). About 0.05 kg of powder of *C. hirsuta* was soaked into 0.5 L of chloroform and 0.5 l 80% methanol in separate flasks. The mixtures in the erlenmeyer flask were placed

on a platform shaker of 120 rpm for 72 h at room temperature (Mohana et al., 2009). Then, the solutions were filtered by Whatman no. 1 filter papers and the solvent extract was concentrated separately under reduced pressure using rotary flash evaporator at 45°C. After complete evaporation of the solvent each the extract was weighed and dissolved in 3% Tween-80 for biological assay.

Anti bacterial susceptibility test determination of leaf extracts of *C. hirsuta*

S. aureus, *E. coli*, *P. aeruginosa*, *S. boydii* and *S. thyphi* were taken from Microbiology Department, Ethiopian Public Health Institute (EPHI), Addis Ababa. The standard bacteria were screened for susceptibility at different doses (50, 100 and 200 mg/ml) of *C. hirsuta* extracts and standard antibiotics ampicillin (30 µg/disk) and chloramphenicol (30 µg/disk). Bacterial broth culture was prepared at a density of 10^8 cells ml⁻¹ which approximately equals to 0.5 McFarland standards. The test microorganisms were uniformly swabbed on the Mueller Hinton Agar (MHA) using the cotton swab. The paper disc diffusion technique was applied to determine the antimicrobial activities of the tested plant extracts. Sterile paper discs (6 mm in diameter) immersed in stock solutions containing 50, 100 and 200 mg/ml prepared in 3% Tween-80 of plant extracts and allowed to dry for 15 min to allow it to diffuse and paper disks were placed on uniformly swabbed agar plate using sterile forceps. The plates were then incubated for 24 h at 37°C and diameters of the inhibition zones were recorded. All tests were carried out in triplicates.

Minimum inhibitory concentration (MIC) determination by agar dilution method

Minimum inhibitory concentration (MIC) was determined by agar dilution methods as described by European society of clinical microbial and infectious diseases (ESCMID, 2000). Nineteen millimeter of molten Mueller Hinton agar (MHA) and 1 ml of extracts from each plant at different concentration (50, 25, 12.5, 6.75 and 3.125 mg/ml) were mixed thoroughly and poured on Petri dish. Petri dishes then were allowed to dry to avoid drops of mixture. The bacterial suspensions in 0.85% saline contains about 1.5×10^8 cells ml⁻¹ colony forming unit (CFU) which were standardized with 0.5 Macfarland. 1 µl of bacterial suspension (approximately 1×10^4 CFU) was inoculated on plate having Muller Hinton agar and extracts as described above. After drying the inoculum spot, the plates were incubated at 37°C 24 h. The MIC was determined by observing the growth bacteria with our naked eyes.

Minimum bactericidal concentration (MIC) determination

Some portions of tests were taken from the (MIC) test plate and were sub-cultured on solid nutrient agar by making streaks on the surface of the agar. The plates were incubated at 37°C for 24 h and the MBCs were determined after 24 h. Plates that did not show growth were considered to be the MBC for the extract.

Oral acute and sub-acute toxicity tests

Oral acute and sub acute toxicity tests were done according to OECD guideline (2001). The Swiss albino mice which were obtained from Ethiopian public health institute (EPHI) and reared in animal house in department of biology, college of natural science, Addis Ababa University were used. The mice were starved for 3 h before the experiment began with only water allowed and 1 to 2 h

Table 1. Percentage extract yield from the leaves of chloroform *C. hirsuta* extracts.

Solvents/plant extract	Dry powder (g)	Solvent (ml)	Ratio (w/v)	Yield (g)	Yield (%)
80% Methanol/ <i>C. hirsuta</i>	50	500	1:10	7.88	15.75
Chloform/ <i>C. hirsuta</i>	50	500	1:10	4.94	9.9

Table 2. The bacterial inhibition zone of leaves extract of *C. hirsuta* on some bacteria (mg/ ml).

Extract/solvent	Dose (mg/ml)	Mean inhibition \pm SEM				
		<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>S. boydii</i>	<i>S. typhi</i>
C.h/Me	50	^a #6.67 \pm 0.01* ^d	^a #6.78 \pm 0.25* ^d	^a #6.37 \pm 0.17* ^d	^b #6.67 \pm 0.02* ^d	^b #6.67 \pm 0.02* ^d
	100	^a #7.5 \pm 0.05* ^d	^a #7.05 \pm 0.06* ^d	^a #7.47 \pm 0.15* ^d	^b #7.82 \pm 0.06* ^d	^b #7.33 \pm 0.05* ^d
	200	^a #9.3 \pm 0.12* ^d	^a #8.5 \pm 0.50* ^d	^a #8.00 \pm 0.29* ^d	^b #9.00 \pm 0.06* ^d	^b #7.90 \pm 0.05* ^d
C.h/Ch	50	^a #7.04 \pm 0.04* ^d	^a #8.40 \pm 0.12* ^d	^a #7.23 \pm 0.02* ^d	^b #7.2 \pm 0.15* ^d	^b #7.33 \pm 0.04* ^d
	100	^a #8.5 \pm 0.05* ^d	^a #9.3 \pm 0.1* ^d	^a #8.31 \pm 0.06* ^d	^b #8.33 \pm 0.02* ^d	^b #8.33 \pm 0.09* ^d
	200	^a #10.7 \pm 0.14* ^d	^a #12.33 \pm 00* ^d	^a #10.26 \pm 0.16* ^d	^b #10.34 \pm 0.13* ^d	^b #11.60 \pm 0.12* ^d
A	30 μ g	12.5 \pm 0.29* ^d	15.33 \pm 0.33* ^d	18.4 \pm 0.29* ^d		
C	30 μ g				23.66 \pm 0.33* ^d	26.83 \pm 0.6* ^d
	T80	00 \pm 00	00 \pm 00	00 \pm 00	00 \pm 00	00 \pm 00

C.h = *Clematis hirsuta*; Me = 80% methanol; Ch = chloroform; A = ampicilin; C = chloroaphinicol; T80 = Tween 80; ^a = A inhibition; ^b = C inhibition; C = chloroamphnicol; # = is significantly higher than; * = is significantly lower than; ^d = T80 inhibition.

after the extracts were given. Female Swiss albino mice, 5 in each group were administered orally with 500, 1000 and 2000 mg/kg body weight from each extract which was dissolved in 3% Tween 80 within 0.2 ml for test and 0.2 ml 3% Tween were administered as control. For acute toxicity studies; gross physical changes within 24 body and weight measured for 14 days. At end of the experiment the experimental mice were anesthesia with chloroform and discarded.

Data analysis

Data was analyzed by using window software; IBM SPSS, version 20. The results were presented as the Mean \pm Standard Error of the Mean (Mean \pm SEM) and statistical significance was considered at a 95% confidence interval (P<0.05). For toxicity test and bacterial sensitivity test was compared by using one way ANOVA and followed by Tukey's test.

RESULTS

The crude extract percentage yield

The highest and minimum yield of the *C. hirsuta* extract obtained from 80% methanol and a chloroform 15.75 and 9.9%, respectively as shown in Table 1.

The bacterial inhibitions of chloroform and 80% methanol extract of *C. hirsuta* are presented as shown in Table 2. The highest inhibition zone (12.33 \pm 0.50 mm) was recorded on *P. aeruginosa* at the concentration (200

mg/ml) whereas; the lowest inhibition zone (6.37 \pm 0.17 mm) was recorded from methanol extract at the concentration (50 mg/ml) on *S. aureus*.

The minimum inhibitory concentration (MIC) of leaves of *C. hirsuta* was determined on five tested bacteria at concentration range of 50 to 1.56 mg/ml as shown in Table 3.

The minimum bactericidal concentration is presented in Table 4. Most bacteria showed their MBC at 6.25 and 12.5 mg/ml.

Acute toxicity test of 80% methanol *C. hirsuta* leaf extract on albino mice

Since 80% methanol *C. hirsuta* leaf extract the highest yield and supposed to dissolve all metabolites dissolved by chloroform, only 80% methanol *C. hirsuta* leaf extract was used to determine oral acute toxicity.

The methanol extracts of the leaves of *C. hirsuta* was administered orally with a single dose of 500, 1000 and 2000 mg/kg body weight to mice.

There were no physical signs such as depression, decrease in feeding activities and hair erection after 80% crude extract administration of *C. hirsuta*. Similarly, there was no mortality in the 14 days follow up after crude extract administration. The weight of the mice increases significantly in each group from day 0 to day 7 and 14 in both extract and water administered mice as shown in

Table 4. Determination of minimum bactericidal concentration (MBC).

Bacterial spp.	Extract	Concentration in mg/ml					
		1.5625	3.125	6.25	12.25	25	50
<i>E. coli</i>	<i>C. hirsuta</i> ^a	+	+	+	+	*	-
	<i>C. hirsuta</i> ^b	+	+	*	-	-	-
<i>P. aeruginosa</i>	<i>C. hirsuta</i> ^a	+	+	*	-	-	-
	<i>C. hirsuta</i> ^b	+	+	*	-	-	-
<i>S. aureus</i>	<i>C. hirsuta</i> ^a	+	+	+	*	-	-
	<i>C. hirsuta</i> ^b	+	+	*	-	-	-
<i>S. boydii</i>	<i>C. hirsuta</i> ^a	+	+	+	*	-	-
	<i>C. hirsuta</i> ^b	+	+	+	*	-	-
<i>S. typhi</i>	<i>C. hirsuta</i> ^a	+	+	+	*	-	-
	<i>C. hirsuta</i> ^b	+	+	+	*	-	-

a, 80% methanol; b, chloroform; +, growth of bacteria; -, no growth; *, minimum inhibitory concentration.

Table 5. Acute toxicity test of 80% methanol *C. hirsuta* leaf extract against body weight.

Group	Dose (mg/kg)	Solvent/extract	Treatment		
			Pre (D0)	Post (D7)	Post (D14)
a	NC	Water	#w2w3 25.97±0.29	#w2 34.77±0.17 ^{*w1}	36.55±0.19 ^{*w1w2}
b	1000	Me/ C.h	#bcw2w3 25.52±0.15	#w3 34.60±0.25 ^{*w1a}	#w3c 35.36±0.16 ^{*w1a}
c	2000	Me/C.h	#bcw2w3 25.80±0.20	#w3c 34.62±0.49 ^{*w1a}	w3 35.33±0.14 ^{*w1a}

NC = negative control; Me = 80% methanol; C.h = *Clematis hirsuta*. a = weight of mice before extract administration; b = weight of mice at 7 days extract administration; c = weight of mice at 14 days extract administration; w1 = weight of mice before water administration; w2 = weight of mice at 7 days water administration; w3 = weight of mice at 14 days of water administration; * = significantly higher weight than; # = significantly lower weight than.

Table 5.

DISCUSSION

Clematis species are known to have different pharmaceutical compounds including glycosides, saponins, alkaloids, xanthenes and anthocyanidins (DaCheng et al., 2013). Anthocyanins is type of flavonoids which can be used as antioxidant activity as hydrogen donating free radical scavengers, anti-inflammatory properties and maintain a healthy heart and urinary tract (Rice-Evans et al., 1996, Prior and Cao, 2000). Researches revealed that alkaloids have pharmacological applied as anesthetics and CNS stimulants (Madziga et al., 2010). Saponin is known to have antiplasmodial activities (Traore et al., 2000). In this research, the crude chloroform extract of the leaves of *C. hirsuta* showed the highest inhibition zone (12.33±0.50 mm) on *P. aeruginosa* at the concentration (200 mg/ml) and followed by *S. typhi* (11.60±0.12 mm), *E. coli* (10.7±0.14 mm), *S. boydii* (10.34±0.13 mm) and *S. aureus* (10.26±0.16 mm),

respectively by agar disk diffusion methods. On the other hand, the methanol extract of *C. hirsuta* leaf at the concentration of 200 mg/ml had 8.00±0.29, 9.3±0.12, 8.5±0.50, 9.00±0.06 and 7.90±0.05 mm inhibition on *S. aureus*, *E. coli*, *P. aeruginosa*, *S. boydii* and *S. typhi*, respectively. Thus, the chloroform extracts of the leaves had significantly higher inhibition zone than the 80% methanol at the same concentration (200 mg/ml). On the other hand, researches in Iran revealed the antimicrobial activities of *C. orientalis* on *S. aureus*, *E. coli* and *P. aeruginosa* with inhibition zone of 11±1.5, 10.3±1.52 and 9.6±0.57 mm, respectively (Raei et al., 2013). As concentration increases from 50 to 200 mg/ml in both extracts, bacterial inhibition zone increased. From this, it is suggested that the plant extracts had dose dependent antibacterial activities.

C. hirsuta leaves also showed a pronounced antifungal activity against the three dermatophytes tested on *T. rubrum*, *M. canis*, and *E. floccosum* and yeast *C. albicans* at the concentration of greater of equal to 46.9 mg/ml (Cos et al., 2002). The inhibition zone of the standards drug chloroamphenicol (30 µg) was interpreted in such a

way that its bacterial inhibition resistant (≤ 12 mm), intermediate (13-17 mm) and sensitive (≥ 18 mm) as described in Bauer et al. (1966). Based on these criteria, the chloroamphenicol inhibition on *S. boydii* (23.66 ± 0.33 mm) and *S. typhi* (26.83 ± 0.6 mm) in this research was more sensitive. In the contrary, the ampicillin inhibition on *S. aureus* *E. coli* *P. aeruginosa* was 18.4 ± 0.29 , 12.5 ± 0.29 and 15.33 ± 0.33 , respectively. The chloroform extract of *C. hirsuta* on *P. aeruginosa* showed the minimum inhibition concentration and minimum bacterial concentration at 3.125 and 6.25, respectively.

C. hirsuta is used to heal respiratory tract problem and cataract in Meinit ethnic group (Giday et al., 2009). This research agreed with traditional healers in that the two plant extracts inhibit the growth of bacteria such as *P. aeruginosa* infecting and affecting the skin. The 80% methanol leaf of *C. hirsuta* in this research (2000 mg/kg) on mice did not reveal any sign symptom and mortality. This result is agreeable to Bhosale et al. (2010). The weight of the mice was also considered as a parameter in the determination of acute toxicity at 500, 1000 and 2000 mg/kg. For this, OECD guideline (2001) was used as a reference for testing the extracts. The weight of mice before treatment significantly increased in both extract and water administered mice at all concentrations on days 7 and 14. This data showed the extracts at or lower 2000 mg/kg are not toxic to mice.

Conclusion

C. hirsuta is one of the major herbal plants used in Ethiopia. Both 80% methanol and chloroform extracts of the leaf of *C. hirsuta* showed antibacterial properties on some pathogenic bacteria (*E. coli*, *P. aeruginosa*, *S. aureus*, *S. boydii* and *S. typhi*) at 50, 100 and 200 mg/ml. Furthermore, the 80% methanol extracts of *C. hirsuta* leaf was not toxic to albino mice at or less than 2000 mg/kg body weight. Thus, this plant is supposed to have antimicrobial properties therefore, further isolation and compound identification is recommended for biological studies.

CONFLICTING INTEREST

The authors declare that they have no conflict of interest.

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