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QUANTITATIVE METHODS FOR THE EVALUATION OF ARTESUNATE IN PHARMACEUTICALS USING N-BROMOSUCCINIMIDE AS OXIDIMETRIC REAGENT

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ABSTRACT
The success recorded so far by the global fight against malaria could be in jeopardy if the distribution of the counterfeit artemisinin derivatives is not checked. One titrimetric and one spectrophotometric method are developed for the assay of artesunate. These methods are based on the use of n-bromosuccinimide (NBS) as the oxidimetric reagent. In the titrimetric method, NBS was titrated directly against artesunate using methyl orange. In the spectrophotometric method a measured excess of NBS was reacted with artesunate, the unreacted NBS is determined using a fixed amount of methyl orange. In both cases, the amount of NBS used is proportional to the drug concentration. In the titrimetric method, the reaction was stoichiometric in the ratio of 1:2 (Drug:NBS); in the range of 1-10 mg/mL.The spectrophotometric method obeyed Beer's law in the range of 0.25-8.0µg/mL.The molar absorptivity was 3.0 x 10^4L/mol./cm and Sandell sensitivity was 0.0095 µg/cm^2. The limit of detection (LOD) and quantification (LOQ) were 0.08µg/mL and 0.17µg/mL respectively. The intra and inter day precision and accuracy ≥ 3.0%. The developed methods were successfully used to assay artesunate tablets procured locally and statistically compared with a reference international pharmacopeia method. The applicability and accuracy were further tested by performing recovery studies via standard addition method with the result showing excellent recoveries confirming that pharmaceutical excipients had no effects on the methods.

KEYWORDS: artesunate, artemisinin derivatives, n-bromosuccinimide, counterfeit, malaria.

INTRODUCTION
As international travels become more and more frequent, malaria is no longer a problem of the tropics, imported malaria is becoming a serious concern. Furthermore, if global warming remains unchecked malaria may reestablish itself in Europe and North America.[1] The concerted effort and well co-ordinated fight against malaria has shown great promise of massive success because of the use of a long lasting insecticide net in the roll back malaria programme. The use of artemisinin combination therapy also contributed immensely to this success story. Unfortunately, this gain could be in jeopardy as there is an emergence of multidrug resistant *Plasmodium* in South East Asia.[2, 3] This resistance is traceable to the manufacture and distribution of counterfeit/fake artemisinin derivatives in South East Asia.[4,5, 6] The counterfeit/fake artemisinin derivatives have also been largely reported in Sub Sahara Africa.[7,8] If the activities of these unscrupulous and criminally minded individuals are not checked, then we may be encountering untreatable malaria. The threat is real and it is a source of serious concern as this may precipitate a serious public health challenge. Artesunate is a hemi succinate water soluble semi synthetic derivative of artemisinin, a sesquiterpene lactone compound obtained from Chinese plant *Artemisia annua.* Artemisinin derivatives have highly potent parasiticidal action against malaria causing *Plasmodium* species. Hence its recommendation by the World Health Organization and authorities of malaria endemic countries.

The manufacturers and distributors of counterfeit artemisinin derivatives are very sophisticated. They produce artemisinin derivatives that look genuine, producing holograms, blister packs showing manufacturing dates and expiry dates that can fool any expert in the field whether pharmacist or government drug control agents.[8] Apart from production of counterfeit/fake artemisinin in a bid to make more profit, substandard artemisinin are also produce using cheap and substandard excipient. Some of the excipient used are potentially dangerous and are known carcinogens such as Safrole, melamines and metamizole.[8] The consumers of these drugs are constantly put in danger. Huge profits are made because counterfeit drugs are usually smuggled and do not pass through the usual port of entry therefore

depriving government of taxes. Finally bearing in mind the huge profit associated with this criminal trade terrorist and organized crime cartels can tap into making the situation worse. To put this nefarious criminal process in check, highly technical laboratories with Hi-tech and modern state of the art equipment are required. These equipment are expensive and are hardly affordable by most of the poor countries where malaria is endemic. Bearing this in mind, the aim of this research work is to develop sensitive, accurate, reproducible and cost effective method that can be used in routine laboratories and field stations by government officials to checkthe influx of these counterfeit artemisinin derivatives especially artesunate. Officially, artesunate is assayed using HPLC and titrimetry (IP 2005).[9] The proposed method could complement the two official methods. Many workers have developed methods for the assay of artesunate.[11,12,13] Some of the methods are quite effective but some also suffer from obvious analytical limitations such as tight pH control, low sensitivity, significant blank absorption, tedious and time consuming process, using organic solvents that are often harmful to the environment and possibly the analyst. Workers have used n-bromosuccinimide for the assay of some pharmaceuticals[14,15] but a careful search of the literature reveals that there is no method yet for the assay of artemisinin derivatives using n-bromosuccinimide (NBS). The two methods as proposed are based on the use of NBS as the oxidimetric reagent. NBS releases low level bromine atom that oxidizes the drug in acid medium.

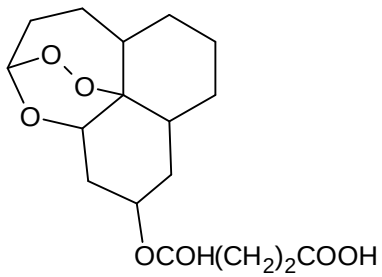


Figure 1. Artesunate.

EXPERIMENTAL

Apparatus: All spectral determinations were made using Spectrophotometer model heylos β Thermo Electron corporation USA with 1cm matched quartz cell.
Chemical and Reagents
All chemicals and reagents used were analytical (Analar) grade.
N-Bromosuccinimide (NBS) A 0.01M solution of NBS was prepared by dissolving approximately 0.20g of the chemical (Sigma,Germany) and dissolved in warm distilled water and diluted to 1liter with distilled water and the resulting solution was standardized iodometrically.
Sulphuric Acid (2M) Concentrated sulphuric acid (Sigma,Germany) was diluted appropriately with distilled water to obtain the concentration of 2M solution.
Methyl Orange (50 µg/mg) First 200µg/mg of methyl orange was prepared by dissolving 23.52mg of the dye (Sigma, Germany) in distilled water and made up to the 100ml mark of the volumetric flask.The resulting solution was diluted to 50µg/ml and used in both methods.
Ethanol 98% ethanol (Sigma, Germany) was used.
Standard Artesunate Solution

The pure artesunate powder was provided by the director of Pharmaceutical Services of the University of Uyo Teaching Hospital as gift. A standard solution was prepared by dissolving 100mg of the pure drug powder in distilled water and made upto 100 ml of the volumetric flask.The resulting solution with a concentration of 1 mg/ml was used for method A and 1ml of the solution was further diluted to obtain a working concentration of 20µg/ml for method B

TITRIMETRIC METHOD

Different aliquots of ART 5-9mL containing 1mg/mL were accurately transferred into 100mL volumetric flask. The volume was made up to 15mL using 98% ethanol. Then 5mL of 2M of sulfuric acid was added and swirled gently to mix well, and titrated against 0.01M NBS; using 2 drops of methyl orange as indicator until the disappearance of red colour at end point. A blank titration was carried out following exactly the same method but carefully omitting the drug. The amount of ART present in each aliquot was determined using the equation
Amount of ART (mg) = VMwtR/n
Where V=volume of NBS used for the complete titration
Mwt=molecular weight of ART (384.4)
R=molar concentration of NBS
n=the number of moles of NBS reacting with ART

Spectrophotometric method

Different aliquots of Artesunte (0.25-5.0) with the concentration of 20 µg/mL were accurately transferred to a series of 10mL calibrated volumetric flask using micro burette. The volumes in the flask were rounded up to 5mL using 98% ethanol. The content of the flask was acidified using 1mL of 2M sulfuric acid followed by 1 mL of 0.01M of NBS and shaken to mix well. The flask was stoppered and allowed to stand for15 minutes with gentle swirling from time to time within the 15 minutes. Finally, 1 mL of 50 µg/mL of methyl orange was added and the volume in the flask was made up to the 10 mL mark and swirled gently and allowed to stand for 5 minutes and the absorbance was measured at 520 nm against a reagent blank prepared in similar manner but without the drug (ART).

A calibration curve absorbance vs drug concentration was plotted and the unknown concentration was deduced from the data obtained from Beer's Law equation.

Procedure for assay of Artesunate tablets

Twenty tablets of artesunate brands procured locally were weighed and powdered using ceramic mortar and pestle. The quantity of the fine powder equivalent to 100mg was weighed and transferred into 100mL capacity volumetric flask containing 20mL of distilled water. The mixture was sonicated for 10 minutes and 50mL of distilled was further added and shaken vigorously for 20 minutes. Finally, the content in the flask was made up to the 100mL mark and shaken further for 5 minutes. The resulting drug mixture was filtered using Whatman filter paper No. 42 discarding the first 10mL of the filtrate, the drug solution with the concentration of 1mg/mL was used for the titrimetric method while a portion of the solution was diluted appropriately to obtain a working concentration of 20 µg/mL from where a suitable aliquot was assayed using the spectrophotometric method.

Assay of placebo blank

A placebo blank of pharmaceutic excipient usually used in the formulation of was prepared as follows: Talc (10mg), Glucose (5mg), Acacia (5mg), Magnesium stearate (5mg), Sodium citrate (5mg), Methylcellulose (10mg), Sodium alginate (5mg), Corn starch was used to bulk the mixture to 100mg. the resulting mixture was homogenized and a solution was prepared as described under the procedure for assay of artesunate tablets.

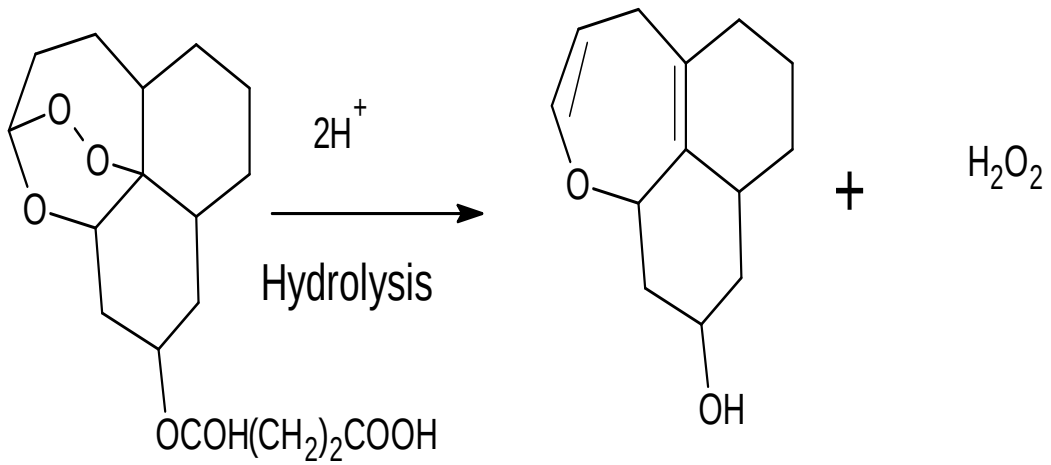
Procedure for the assay of artesunate in synthetic mixture

About100mg of the pure artesunate powder was carefully measured out and mixed with 100mg of placebo blank powder prepared exactly as above. The two powders were homogenized and mixed appropriately then 100mg of the synthetic mixture was measured out and carefully transferred into 100mL capacity volumetric flask containing 30mL of distilled water and sonicated for 10 minutes and further shaken vigorously after the addition of 20mL of distilled water. The resulting solution was made up to the 100mL mark using distilled water and shaken further. The mixture was then filtered using Whatman filter paper No. 42, the first 10mL of the filtrate was discarded. The resulting solution with concentration of 1mg/mL obtained and a suitable aliquot was assayed using the described titrimetric method. Another part of the solution was taken and diluted appropriately with distilled water to obtain a working concentration of 50 µg/mL from where a suitable aliquot was analyzed using the spectrophotometric method.

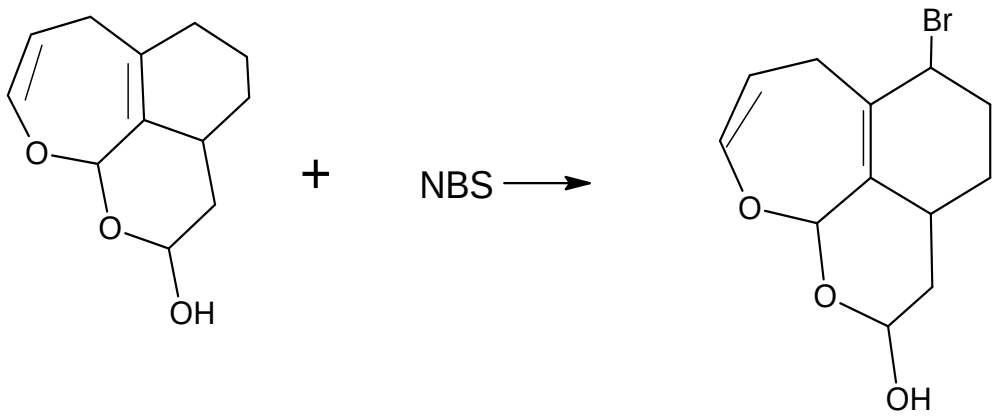
RESULTS AND DISCUSSION

NBS is an oxidizing agent. It generates and releases positive bromine in chemical reaction. It is used as a brominating agent. It is specifically used for the bromination of alkenes at the allylic position.[16] A careful search of the literature shows that NBS has been used in the determination of several pharmaceuticals.[14,15] To the best of our knowledge, NBS has not been used to assay or determine any of the artemisinin derivatives.

In the present work (a) in the titrimetric method NBS was titrated against the artesunate and the reaction was stoichiometric. Methyl orange was used as the indicator for the reaction. In the second method, spectrophotometry, a known excess of NBS was made to react with the drug (Artesunate). This reaction was allowed to proceed to conclusion after a set time, then the surplus NBS was determined using methyl orange. The mechanism of reaction of the process is not well understood but there is a strong suggestion that point towards the fact that allylic positions are created in the Artesunate molecule under acid condition. In this acid condition, the endoperoxide bond of the artesunate molecule is cleaved generating hydrogen peroxide and also creating allylic position as such shown.



Scheme 1. In acid medium the endoperoxide bond is cleaved generating hydrogen peroxide in situ and creation of allylic centers in the resulting molecule.



Scheme 2.The active bromine produced by the NBS attaches at the allylic position created in the resulting molecule.

The hydrogen peroxide generated *in situ* also reacted with the positive bromine released by the NBS. The dye specifically is oxidatively destroyed. Hydrobromic acid formed from the reaction of bromine and hydrogen peroxide oxidatively destroys the dye methyl orange.

Of the two mechanism of reaction, the most acceptable is that NBS is a specific reagent for effecting the allylic bromination of alkenes. The reaction is a free radical reaction requiring peroxide or light as initiators.[17] The hydrogen peroxide required as initiator is generated *in situ* as a result of the cleaving of the endoperoxide bond of Artesunate in acid medium. Since the reaction of the bromine liberated by NBS requires H_2O_2 as initiator of the free radical reaction. The H2O2 initiates the abstraction of hydrogen from allylic position as shown in the reaction scheme. Finally, bromine is attached to the allylic position created.

Titrimetric method

This is a direct titrimetric method in which case artesunate was titrated directly against NBS in acid medium (H_2SO_4) using methyl orange as indicator. The reaction was found to be stoichiometric and the ratio was 1:2 (ART:NBS). Quantitative result were obtained when H_2SO_4 was used; 5mL of 2M $H_2SO_4$was adequate in 20mL reaction volume. Slight increase or decrease in the volume of the H_2SO_4 (i.e. up to 6mL or 4mL) did not affect the stoichiometry of the reaction. Hydrochloric acid was also used to acidify the reaction. It was discovered that 5mL of 2M HCl was equally good for the reaction process. The reaction was instantaneous and proceeded well under normal room temperature of 25C±1. There was no need to increase temperature.

Spectrophotometric method

The methyl orange was oxidativelydestroyed by NBS. In other to determine limits and the concentration of the methyl orange that could be destroyed by NBS. Initial experiments were performed which showed that 50µg/mL of methyl orange was required in a reaction volume of 10mL. The reaction was instantaneous. There was no need to increase the reaction temperature as the room temperature was adequate. Sulfuric acid was adequate for the acidification of the process. 1mL of 2M H_2SO_4 was found to be adequate in a 10mL reaction volume. The oxidation process was complete in 5 minutes; contact time of up to 15 minutes did not affects the stoichiometry of the reaction.

METHODS VALIDATION

The methods as proposed were validated for linearity, sensitivity, accuracy and precision, selectivity and recovery.

Linearity and Sensitivity

In the titrimetric method the linear range was 5-9mL and reaction was quantitative and stoichiometric in the ratio of 1:2 for ART:NBS within this range. In the spectrophotometric method, the calibration curve generated was linear as obtained via the least square method. Hence, it obeyed Beer's law; with the linear range of 0.25-5mg/mL. The calibration graph was described by the equation

$A = BC + D$

Where A is absorbance, B the intercept, C the concentration and D the intercept. The Sensitivity parameters which include Molar absorptivity, Sandell sensitivity, limit of detection (LOD) and limit of quantification (LOQ) were determined as per the current ICH guidelines (International Committee on Harmonization 2005) using the formular

$LOD = 3.3\sigma/s$, $LOQ = 10\sigma/s$

Where σ is the standard deviation for five reagent blank determination and s is the slope of the calibration graph. Both values for linearity and sensitivity of are recorded in table 2.

Precision and Accuracy

The precision and accuracy of this developed methods were determined by performing six replicate analysis each at 3 concentration levels. This was done within same day (intra-day) and 5 consecutive days in a week (inter-day). Percentage relative standard deviation %RSD was used to evaluate precision with percentage

relative error %RE was used to evaluate accuracy. The % RE was calculated using the formular

$$\%RE = \frac{[\text{amount found} - \text{amount taken}] \times 100}{\text{Amount taken}}$$

The calculation values obtain were compared with the theoretical values and rendered in table 3.

Selectivity: the selectivity of the proposed method were evaluated via placebo blank and synthetic mixture as described earlier. Both placebo and synthetic mixture were analyzed using the proposed methods as described. In both cases there were no reasonable interference from excipient used when tablets are formulated. The recoveries of the pure drug in the synthetic mixture ranged from 94.58 to 120.80 with standard deviation of 1.20-1.67. See table 4.

Application of the method to assay tablets

The developed methods were successfully used to evaluate 4 commercial brands of Artesunate tablets purchased from local pharmacies within Uyo metropolis in South South Nigeria. The result obtained were statistically compared with an official method (International Pharmacopoeia 2005-Titrimetric method) via student-t test and F-test (Variance ratio) at 95% confidence level and at 4 degrees of freedom. In all cases the result showed that the calculated values were below the critical (tabulated) values. The result showed no significant difference with those obtained when from the Pharmacopoeial method.

To ascertain the accuracy and practicability of the methods, recovery studies was performed via standard addition method. In this case a pre-analyzed tablet powder was spiked with pure ART at 3 different concentration level and the total was determined by the proposed methods. The recoveries of the added pure ART powder was found to be $\geq 98 \leq 104\%$ with standard deviation of 1.15-0.83 as recorded in table 5. Showing excellent recoveries meaning that pharmaceutical excipients had no interference with the developed method.

Table1. Sensitivity and regression parameters

Parameter	Value
λ_{max}	520
Linear range (µg/mL) (Beer's Law Range)	0.25-8.0
Molar absorbtivity (L/mol/cm)	3.8×10^4
Sandell sensitivity (µ/cm^2)	
Limit of detection (LOD) µg/mL	0.08
Limit of Quantification (LOQ)	0.17
Regression equation	A = BC + D
Slope	0.0127
Intercept	0.0001
Correlation coefficient	0.9998

Recovery studies

Table 2 Evaluation of inter-day and intra-day accuracy and precision

Method	ART Taken (mg)	Intra-day accuracy and precision			Inter-day accuracy and precision		
		ART found (mg)	RE%	RSD%	ART found (mg)	RE%	RSD%
Titrimetry (mg/mL)	2.0	2.04	2.0	0.50	2.05	2.5	1.22
	4.0	4.10	2.5	1.06	4.10	2.5	1.02
	6.0	6.15	2.5	1.22	6.17	2.8	1.26
Spectrophotometry (µg/mL)	30	30.86	2.9	1.28	2.85	2.80	1.37
	60	61.50	2.5	0.94	61.7	2.80	1.70
	90	92.00	2.2	1.09	92.2	2.40	1.09

Table 3: Results of Analysis of Tablets Using the Proposed Methods

Commercial Artesunate tablets analyzed	Label claim (mg)	Found (percent of label claims+SD)		
		Reference	Method "A" Titrimetry	Method "B" Spectrophotometry
Artesunate (Sanofi)	50	99.70 ± 1.37	101 ± 1.02 F=1.80 t=2.19	102±1.05 F=1.70 t=1.75
Lever Artesunate (Geneith)	50	99.80 ± 1.20	101.32 ± 1.30 F=1.17 t=1.07	101.7 ± 1.28 F=1.14, t=1.36
Articin (Evans)	50	99.72 ± 1.50	103.10 ± 1.60 F=1.14 t=1.54	102 ± 1.43 F=1.10 t=1.16
Artesunate (Neros)	50	99.72 ± 1.43	102.20 ± 1.76	101.4 ± 1.24

			F=1.51 t=1.08	F=2.02 t=0.99

Table 4: Results of Recovery Study via Standard Addition Method

Drug formulation studied	Titrimetry				Spectrometry			
	Amt of ART in tablet (mg/mL)	Amt of pure ART added (mg/mL)	Total amount found	%recovey of pure ART+SD	Amt of ART in tablet (mg/mL)	Amt of pure ARTadded (mg/mL)	Total amount found	%recovery of pure ART +SD
Artesunate (Sanofi)	3.05	3.0	3.07	101.0 ±1.10	3.05	3.0	6.04	99.6 ±1.30
	3.05	6.0	9.09	101.3 ±1.20	3.05	6.0	9.06	100.3 ±1.50
	3.05	9.0	12.12	102.3 ±1.11	3.05	9.0	12.1	101.6 ±1.16
Lever Artesunate (Geneith)	4.09	3.0	4.11	100.5 ±1.06	4.09	3.0	7.08	99.0 ±1.18
	4.09	6.0	10.12	101 ±0.76	4.09	6.0	10.11	100.5 ±1.10
	4.09	9.0	13.20	110. ±1.0	4.09	9.0	13.08	99.8 ±1.17
Articin (Evans)	5.03	3.0	8.12	102. ±1.12	5.03	3.0	8.10	101.3 ±1.11
	5.03	6.0	11.14	102. ±1.16	5.03	6.0	11.09	101.2 ±1.10
	5.03	9.0	14.13	102 ±1.20	5.03	9.0	14.02	99.8 ±1.20
Artesunate (Neros)	6.03	3.0	9.08	101 ±0.76	6.03	3.0	9.02	99.8 ±1.14
	6.03	6.0	12.02	99.8 ±1.3	6.03	6.0	12.10	101.2 ±1.12
	6.03	9.0	15.01	99.7±1.5	6.03	9.0	15.13	101.7 ±0.81

Mean value of 3 determinations

CONCLUSION

Two new methods are developed for the assay of ART in tablets and bulk. The methods are sensitive, accurate and precise. They are reproducible requiring no tedious extraction using hazardous organic solvent and no tight pH control. The methods are recommended routine laboratory and field station analyses of ART. The analyst and the environment face no hazard because of the reagents used.

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REFERENCES

1. Drew M.G.B., Metcalf J., Dascombe M.J and Ismail F.M.D. Reactions of artemisinin and arteether with acid : Implication for stability and mode of action. *Journal of Medicinal Chemistry*, 2006; 49: 6065-6073.
2. World Health Organization, Global malaria Programme. (2014).Status report on artemisinin resistance .Global malaria programme WHO/HTM:GMP/2014-9.
3. World Health Organization (2013) Emergency response response to artemisini resistance Greater Mekong sub region. http//apps.who.int/bitstream/10665/79940/1/9787241505321.eng pdf.
4. Rozendal J . Fake antimalarial drugs in Cambodia, *Lancet.*, 2001; 357(923): 890.
5. Newton , P.N., Dondorp p., Green M.D., Nayyar ,M and Green N. J., Counterfeit artesunate antimalaria in Southeast Asia. *Lancet.*, 2003; 362(9378): 169.
6. Newton P. N,. Proux S., Green M.D., Smithius F and Rozendaal J. Fake artesunate antimalarial in Southeast Asia. *Lancet.*, 2001; 373: 1948 – 1950.
7. Atemkeng M.A., DeCock K.U and Plaizer – VerCammen J. Quality control of active ingredients in artemisini derivatives within DR Congo. *Journal of Tropical Medicine and International Health*., 2007; 12(1): 68 - 74.
8. Nayyar, G M L, Breman JG., Newton P.N., and Herrington J; Poor quality antimarial drugs in Southeast Asia and Sub Saharan Africa. *The Lancet Infectious Disease.*, 2012; 12(6): 488-496.
9. Ambroise – Thomas, P.D. The tragedy caused by fake antimalarial drugs. *Mediterranean Journal of Hematology and Infectious disease.*, 2012; 4: 2012027.
10. International Pharmacopoiea 3rd Edition, 2003; 217.
11. Green M.D., Mount et al. Authentication of artemether, Artesunate and dihydroartemisinin tablets Using simple colorimetric method. *Tropical Medicine and International Medicine*, 2001; 6(12): 980 – 982.
12. Gabriels M and Plaizer – Vercammen J. Development of revers phase thin layer chromatographic method for artemisini and its derivatives. *Science*, 42(7): 341 – 347.
13. Adegoke O. A and Osoye A. O. Derivatization of artesunate and dihydroartemisinin for colorimetric analysis using P-dimethylaminobenzaldehyde. *Eurasian Journal of analytical Chemistry*. 2011; 6(2) 104 – 113. 17.
14. Basavaiah K and Anil Kumar UR. Optimised and validated spectrophotometric methods for the

determination of Gatifloxacin in Pharmaceuticals using N- Bromosuccinimide and three dyes. *Proc. Indian Nat Sci Acad.*, 2006; 4: 225- 229.

15. Vinay K.B., Revanasiddappa H,D., Devi O.Z., Ramesh P.I. and Bsavaiah k. Rapid titrimetric and spectrophotometric determination of Ofloxacin in Pharmaceuticals using N-Bromosuccinimide. *Brazillian Journal of Pharmaceutical Sciences.*, 2011; 47 (2)
16. Morrison R.T., and Boyd R.N. Organic Chemistry 6th Edition, New Jersey ;Printice – Hall 2007 pp. 390.
17. Allinger N., Cava M., Jongh D., Johnson C., Lebel N., and Stevens C.Organic Chemistry Textbook. 1st Edition. Worth Publishers INC. New York., 1971; 590 & 639.