

Original Research Article

Evaluation of *Vigna subterranea* seed extract as possible adjuvant for Typhoid fever vaccine

ABSTRACT

Typhoid fever is an important communicable disease that is endemic in developing countries characterized by poor water supply, sanitary conditions and increasing population. Vaccination with the *Salmonella typhi* (ST) vaccine is an effective control measure. The two currently licensed ST vaccines are saddled with a lot of drawbacks which can be overcome by the addition of adjuvant. *Vigna subterranea* (VS) ethanolic extract was evaluated as a possible adjuvant to ST vaccine. Mice were vaccinated with typhoid vaccine followed by daily weight measurement, and enumeration of microbial colony counts post-vaccination challenge. The blood culture results showed that mice group that received our formulated vaccine had the lowest microbial load (82.2 ± 29.72) following microbial challenge, the mice body weight assessment results also emphasize this, as the mice in this treatment group had an uninterrupted healthy growth. Further, from our results VS extract demonstrated a relative inability to function as effective adjuvant to the ST vaccine since the microbial load in this group (vaccine + VS extract) was high (152.3 ± 47.67) hence the ineffectiveness of combining both agents.

1 INTRODUCTION

Typhoid fever, a systemic infection caused by *Salmonella enterica* serotype *Typhi* (*S. typhi*) remains an important public health problem globally as it is a major cause of death in the developing countries [1]. In 2000, it was estimated that over 2.16 million episodes of typhoid occurred worldwide, resulting in 216 000 deaths [1]. The infection is commonly acquired from water or food contaminated by the feces of an infected person. The incubation period is 6–30 days, and illness onset is insidious, characterized with symptoms such as gradual onset of sustained fever, chills and abdominal pain. In some cases, patients experience rash, nausea, anorexia, diarrhea or constipation, headache, relative bradycardia and reduced level of consciousness [2]. Patients with untreated typhoid fever are reported to have case-fatality rates >10% [2] while those with early and appropriate antibiotic treatment is typically <1% [3]. Although improved water quality and sanitation constitute ultimate solutions to this problem, vaccination in high-risk areas is a potential control strategy recommended by WHO for the short-to-intermediate term [4]. This importance of vaccination is heightened by the increasing resistance of the aetiological agent to antimicrobial agents, including fluoroquinolones, in many parts of the world [5]. In 2003, the World Health Organization recommended strategies to reduce typhoid fever which are routine immunization of school-aged children, immunization of children beginning at 2 years of age in sites where typhoid fever occurs in younger children [4] and immunization of travellers going to endemic areas. Two safe and efficacious typhoid vaccines, the injectable Vi capsular polysaccharide vaccine (Typhim Vi, manufactured by Sanofi Pasteur) and the oral live attenuated vaccine (Vivotif,

manufactured from the Ty21a strain of *Salmonella* serotype Typhi by PaxVax), have been licensed; and new, improved candidate vaccines are currently being tested [6-8]. The Vi polysaccharide vaccine is administered as a single injection and is approved for adults and children aged ≥ 2 years and is given at least 2 weeks before potential exposure. The oral Ty21a vaccine is administered in 4 doses on alternating days over 1 week and is approved for adults and children aged ≥ 6 years, this is usually administered 1 week before potential exposure. Repeat doses of typhoid vaccine are needed to maintain immunity, when repeated exposure to the *Salmonella typhi* is expected. An optimal revaccination schedule for the Vi polysaccharide vaccine has not been established; however, the manufacturer recommends a repeat dose every 2 years after the primary dose if continued or renewed exposure is expected [9]. The manufacturer of Ty21a recommends revaccination with the entire 4-dose series every 5 years if continued or renewed exposure to *Salmonella* serotype Typhi is expected [10]. An effective adjuvant can eliminate the need for revaccination or at least ensure revaccination at a longer time interval. Additionally, a suitable adjuvant can equally allow immunization with fewer doses of this oral vaccine, enable reductions in the quantity of antigen contained in an individual vaccine dose, and can be used to increase the response to this vaccine especially in the under-aged population [10-12].

Vigna subterranea (L.) Verdc also known as Bambara groundnut, Bambara bean, "Okpa", earth pea, Congo goober, hog-peanut or ground bean to natives is evaluated as an adjuvant to typhoid fever vaccine. This legume, whose origin has been traced back to Africa, is the third important legume after groundnut (*Arachis hypogea*) and cowpea (*Vigna unguiculata*) [13] however, it is one of the underutilized crops [14]. It is highly nutritious, and has been termed a complete food. Its seed consist of 50–65% carbohydrate, 15–20% protein, 4–9% fat, 3–5% fiber, 3.2–4.4% ash and 2% mineral [15]. *Vigna subterranea* (VS) has antioxidant and antimicrobial properties [15-18] thus might be a useful immunological adjuvant.

Keywords: Vaccine, *Salmonella typhi*, immunogenicity, *Vigna subterranea*, colony forming units.

2 MATERIAL AND METHOD

2.1 Plant collection, identification and extraction

VS seeds were purchased from Ogige market in Nsukka area of Enugu State, Nigeria and authenticated by a botanist from the Department of Botany, University of Nigeria, Nsukka. This was pulverized and the powder obtained was stored in an air tight container until extraction. Ethanolic extract of the fine powder was obtained using cold maceration technique [19] in which 250g of powder was macerated with one (1) liters of absolute ethanol in a glass jar. The preparation was sealed properly to avoid evaporation. After 24 hours, the extract was filtered using a muslin cloth and Whatman No. 1 filter paper. The filtrate obtained was evaporated to dryness at room temperature.

2.2 Test Animals

Twenty-five (25) young female Swiss albino mice (8 - 9 weeks old) purchased from the Faculty of Veterinary Medicine, University of Nigeria Nsukka and kept under standard pathogen-free conditions in an animal facility of the department of pharmacology and toxicology of the University of Nigeria, Nsukka were used. These animals were fed with standard feed and water *ad libitum* throughout the study period. The use and care of the laboratory animals in this present study was done in accordance with the ethical guidelines of the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka.

89 **2.3 Typhoid fever vaccine**

90 The vaccine used was prepared locally by heat denaturation method.

91 **2.3.1 Collection and isolation of *Salmonella typhi* (ST)**

92 ST was obtained from the University of Nigeria Teaching Hospital, Ituku-Ozalla Enugu.
 93 Pure culture of the isolate was prepared by subculturing into freshly prepared Salmonella-
 94 shigella agar by streak method and then incubated at 37°C for 24 hours. After incubation,
 95 distinct colonies were again transferred/subcultured into freshly prepared and sterilized
 96 nutrient broth and incubated for another 24 hours.

97 **2.3.2 Determination of bioload**

98 Exactly 1ml of the broth culture of the microorganism ST was collected and transferred
 99 aseptically into a test tube containing 9ml of sterile water and this was tagged 10^{-1} (10-fold
 100 serial dilution); this was continued to the ninth test tube (10^{-9}) and the last 1ml (i.e. from this
 101 test tube) transferred into a beaker to be discarded appropriately. Nine well-labeled nutrient
 102 agar plates corresponding to the nine test tubes (10^{-1} to 10^{-9}) were each divided into 8 sections
 103 and from each test tube/dilution (e.g. 10^{-2}), one drop each on the 8 sections were made (a total
 104 of 8 drops of that same dilution) on the corresponding nutrient agar plate. The plates were
 105 then incubated at 37°C for 24 hours, after which the viable cell count (using an appropriate
 106 dilution, i.e. one that is clear enough to be counted) was done to determine the bioload or
 107 concentration of the microorganism.

108 **2.3.3 Determination of death time of the microorganism**

109 A loopful was transferred from the selected dilution test tube at time 0, into a recovery test
 110 tube containing nutrient broth. The test tube with its content was then heated (at a
 111 temperature of about 55-60°C) and at various time intervals (i.e. 10, 20, 30, 40, 50, 60, 70,
 112 80, 90minutes), subculturing into the respective recovery test tubes (labeled according to the
 113 time). The recovery test tubes were then incubated for 48 hours at 37°C, after which the test
 114 tubes were examined for microbial growth (indicated by turbidity) so as to determine the
 115 death time.

116 **2.3.4 Formulation of ST vaccine**

117 The selected broth culture was centrifuged at 3000revolutions per minute for 5 minutes. The
 118 supernatant was then removed with the aid of the micropipette and the cells washed twice
 119 with normal saline by centrifuging and removing the supernatant in each case. The cells were
 120 then re-suspended in a specified volume of normal saline (5ml) and heated (at the same
 121 temperature) for a period of time equivalent to the predetermined death time. The vaccine,
 122 thus formulated, was then aseptically transferred into bijou bottles and made up with normal
 123 saline, labeled appropriately and stored in the refrigerator.

124 **2.4 Vaccination**

125 The experimental animal were divided into five groups named A, B, C, D and E of five mice
 126 each and vaccinated intraperitoneally as follows; Group A received absolute ethanol (0.4ml)
 127 only (solvent used in extraction), Group B received 500mg of the VS extract /kg body weight
 128 only, Group C received normal saline only (solvent used in vaccine constitution), Group D
 129 received 0.4ml of the vaccine only (which contains 10^8 cells). Group E received 0.4ml of the
 130 vaccine and 500mg of VS extract/kg body weight of the mice. The vaccination was repeated

131 once after two weeks. Blood samples were collected from the mice by intraocular eye
132 puncture using the method described by [13] at 1 week and 2 weeks post first vaccination and
133 1 week post second vaccination.

134 **2.5 Weight monitoring of the experimental animals**

135 From day 1 post second vaccination, each of the experimental animals was weighed on a
136 daily basis using a digital sensitive weighing balance and the weight recorded accordingly.
137 This continued till the animals were sacrificed.

138 **2.6 Challenge of animals with live ST**

139 After the 1 week post second vaccination blood sample collection, the experimental animals
140 were challenged on the 3rd day with 10⁷ live ST organisms contained in 0.04ml preparation
141 through the intraperitoneal route.

142 **2.7 Blood culture**

143 Four days post challenge, blood samples were collected from each of the mice and 2 fold
144 serial dilutions of the blood samples were done by diluting 25μL of the blood sample with
145 25μL of normal saline. 10μL of each of the diluted blood samples was then aseptically placed
146 on each of the respective portions of the properly labeled nutrient agar plates. This was
147 carried out for all the mice. The plates with its contents were incubated for 24 hours at 37°C
148 and then examined for growth of microorganisms via colony count.

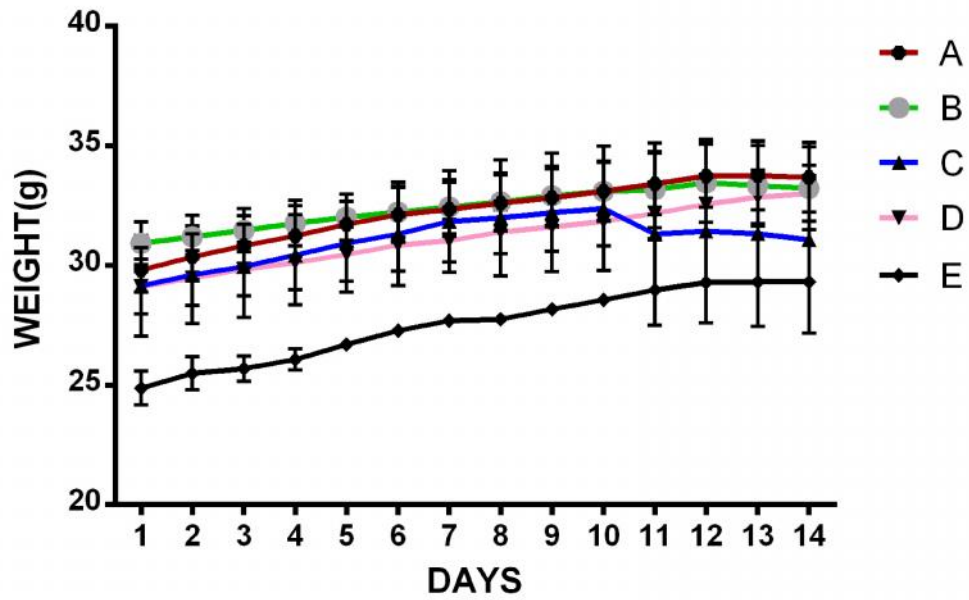
149 **2.8 Statistical analysis**

150 The data obtained was expressed as mean ± standard error of mean (Mean ± SEM). One way
151 analysis of variance (ANOVA) followed by Tukey's post hoc test were used to test for
152 significance. $P < 0.05$ was considered significant. Graph pad prism (version 6.0) was used for
153 the analysis.

154 **3 RESULTS**

155 **3.1 Mice body weight**

156 From the periodic mice body weight monitoring, the mice were seen to grow progressively in
157 all the groups except the normal saline treated group which had a decline from the 10th day
158 (Fig 1-6).



159

160 **Fig 1: Mice body weight curve**

161 A= Ethanol

162 B= VS (500mg)

163 C= Normal saline

164 D= ST vaccine (0.4ml = 10⁸cells)

165 E= ST vaccine (0.4ml =10⁸cells) + VS (500mg)

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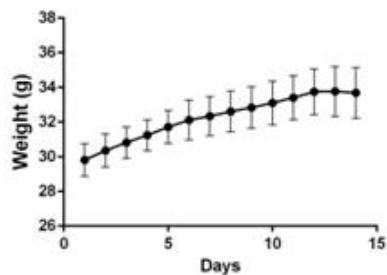


Fig 2: Weight curve of Ethanol treated group

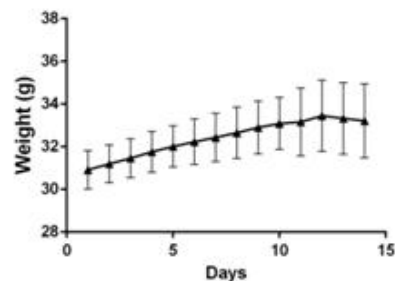


Fig 3: Weight curve of *Vigna subterranea* (500mg) treated group

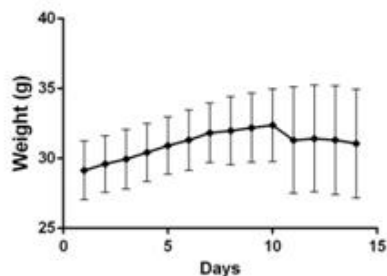


Fig 4: Weight curve of Normal saline treated group

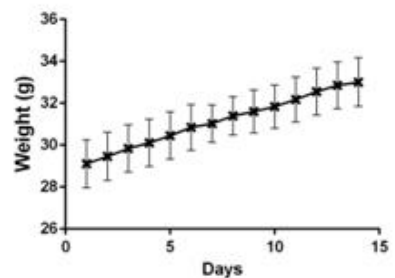


Fig 5: Weight curve of *Salmonella typhi* vaccine treated group

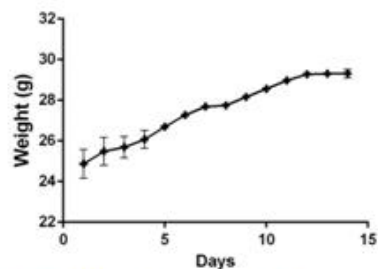


Fig 6: Weight curve of *Salmonella typhi* vaccine + *Vigna subterranea* (500mg) treated group

3.2 Blood culture

From the blood culture, distinct colony forming unit were seen which were counted and recorded as shown in Fig 7.

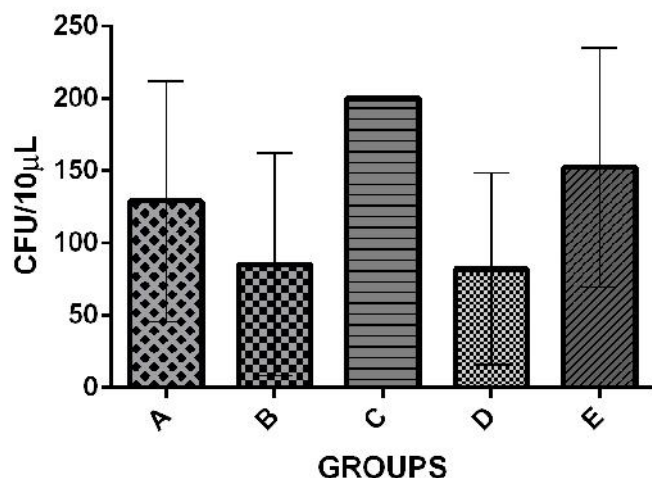


Fig 7: A graphical representation of microbial count following blood culture

A= Ethanol
 B= VS (500mg)
 C= Normal saline
 D= ST vaccine (0.4ml = 10^8 cells)
 E= ST vaccine (0.4ml = 10^8 cells) + VS (500mg)

4 DISCUSSION

Animal body weight monitoring is important in research studies, as it provides information as regards the health status of the experimental animal especially if infection or a disease state is involved [20]. ST infection is known to be associated with significant weight loss [1] hence the need for weight monitoring of the mice in the different treatment groups. The weight assessment results showed a pronounced decrease in weight after challenge for the normal saline treated group C (Fig 4) compared to other groups this is attributed to the fact that normal saline has no antimicrobial, prophylactic and therapeutic effects thus serving as control, it equally shows that the organism administered during challenge are live and viable ST. The ST vaccine group D (Fig 5) had an uninterrupted growth despite the challenge this goes a long way to buttress the observation that our laboratory-prepared vaccine is efficacious, thus offering immunity to the infection. The (vaccine + adjuvant) group E (Fig 6) also had a progressive growth after the challenge possibly because of the vaccine present in the formulation. The ethanol-treated group A (Fig 2) although slightly interrupted at day 12 equally had a progressive growth. This progressive growth despite the microbial challenge can be attributed to the inherent effect of ethanol which could have provided a sterilizing effect (antimicrobial activity) which may have inhibited the growth of the ST administered during the challenge. The VS treated group B (Fig 3) also had a slightly disturbed progressive growth. The antimicrobial properties of this plant could provide an explanation of the limitation posed to ST invasiveness in the group of mice hence the positive growth curve observed for the mice.

The blood culture results (Fig 7) shows the relative performnace of the various treatment administered as prophylaxes against ST challenge. The outcome further supports the results obtained from the periodic mice body weight assessment. The very high microbial load (200 ± 0 CFU) seen in the normal saline treated group is expected since it is the negative control substance lacking antimicrobial, prophylactic and therapeutic potentials. The vaccine-treated group had the lowest microbial count (82.2 ± 29.72 CFU) because of the prophylactic effect of the formulated vaccine. The (vaccine + VS) group altough had a relatively high microbial load (152.3 ± 47.67 CFU), it is however less than that of the normal saline control group possibly because of the vaccine presence. However, the microbial load in this group is higher than what was seen in the group treated with vaccine only, suggesting that VS may not serve as an effective adjuvant to typhoid fever vaccine. Surprisingly, the VS-treated group B recorded a low microbial count of 85.5 ± 38.52 CFU, and this could be attributed to the antimicrobial effect of the plant [15-18]. It is of course understandable that the ethanol solvent (although possessing antimicrobial properties) could not prevent the growth of the challenge ST organism in the mice since the amount administered (0.4ml) is very small. Hence, a relatively high microbial load of 129.0 ± 41.51 was recorded. It is not very clear why this was not reflected in the mice body weight. This disconnetion may be as a result of the robust immune status of the mice used in this group which prevented such weight loss outcome. Nevertheless, microbial enumeration in blood culture of challenged animals would serve as a more specific assessment of the effectiveness of the administered treatments viz-a-viz the microorganisms present in an animal host system.

5 CONCLUSION

Our work has evaluated the potential of VS as adjuvant for ST vaccine. Our findings showed that the combination of VS and ST vaccine demonstrated improvement clinical outcome in comparison to the negative control (normal saline) only, but not to the ST vaccine-alone group. This result reflects the relative inefficacy of combining both agents. Therefore, the need for continuous search for adjuvants to ST vaccine to boost the immune response against ST infection is recommended.

REFERENCES

1. Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. Bull World Health Organ. 2004; 82: 34653.
2. Stuart BM, Pullen RL. Typhoid: clinical analysis of 360 cases. Arch Intern Med (Chic). 1946; 78: 629-661.
3. Bhan MK, Bahl R, Bhatnagar S. Typhoid and paratyphoid fever. Lancet 2005; 366:749–762.
4. WHO. Background document WHO/V&B/03.07: The diagnosis, treatment and prevention of typhoid fever. Geneva: WHO; 2003.
5. Crump JA, Mintz ED. Global trends in typhoid and paratyphoid Fever. Clin Infect Dis 2010; 50:241–246.
6. Acharya IL, Lowe CU, Thapa R, Gurubacharva VL, Shrestha MB, Cadoz M, et al. Prevention of typhoid fever in Nepal with the VI capsular polysaccharide of *Salmonella typhi*. A preliminary report. N Engl J Med. 1987; 317:1101-1104.

- 248 7. Acosta CJ, Galindo CM, Ochiai RL, Danovaro-Holliday MC, Page AL, et al. The role
249 of epidemiology in the introduction of VI polysaccharide typhoid fever vaccines in
250 Asia. J Health Popul Nutr. 2004; 22:240-245.
- 251 8. Simanjuntak CH, Paleologo FP, Punjabi NH, Darmowigoto R, Soeprawoto,
252 Totosudirjo H, et al. Oral immunisation against typhoid fever in Indonesia with Ty21a
253 vaccine. Lancet. 1991; 338:1055-1059.
- 254 9. Typhim Vi Polysaccharide Vaccine [package insert]. Available at [http://](http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM142811.pdf)
255 [www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/](http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM142811.pdf)
256 [ApprovedProducts/UCM142811.pdf](http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM142811.pdf).
- 257 10. Vivotif Typhoid Vaccine Live Oral Ty21a [package insert]. Available at
258 [http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/](http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM142807.pdf)
259 [ApprovedProducts/UCM142807.pdf](http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM142807.pdf)
- 260 11. Banzhoff A, Gasparini R, Laghi-Pasini F, Staniscia T, Durando P, Montomoli E, et al.
261 MF59-adjuvanted H5N1 vaccine induces immunologic memory and heterotypic
262 antibody responses in non-elderly and elderly adults. PLoS ONE. 2009; 4:e4384.
- 263 12. Onah IA, Onukwube GI, Odoh CE, Odimegwu DC. *Moringa oleifera*, an adjuvant for
264 respiratory syncytial virus vaccine. Aust J Basic & Appl Sci. 2017; 11(12): 95-101.
- 265 13. Ntundu WH, Bach IC, Christiansen JL, Andersen SB. Analysis of genetic diversity in
266 bambara groundnut [*Vigna subterranea* (L) verdc] landraces using amplified
267 fragment length polymorphism (AFLP) markers. Afr J of biotech. 2004; 3(4): 220-
268 225.
- 269 14. Bamishaiye OM, Adegbola JA, Bamishaiye EI. Bambara groundnut: An under-
270 utilized nut in Africa. Adv in Agric Biotech. 2011; 60-72.
- 271 15. Koné M, Paice AG, Touré Y. Bambara groundnut [*Vigna subterranea* (L.)Verdc.
272 (Fabaceae)] usage in human health. In: Nuts and Seeds in Health and Disease
273 Prevention (edited by V.R. Preedy, R.R. Watson & V.B. Patel). UK: Elsevier Inc.
274 2011; 192-194.
- 275 16. Pale E, Nacro M, Vanhaelen M, Vanhaelen-Fastré. Anthocyanins from Bambara
276 Groundnut (*Vigna subterranea*). J of Agric and Food Chem. 1997; 45:3359-3361.
- 277 17. Chen AY, Chen YC. A Review of the Dietary Flavonoid, Kaempferol on Human
278 Health and Cancer Chemoprevention. Food Chem. 2013;. 138(4):2099-2107.
- 279 18. Brough SH, Azam-Ali SN, Taylor AJ. The Potential of Bambara Groundnut (*Vigna*
280 *subterranea*) in Vegetable Milk Production and Basic Protein Functionality Systems.
281 Food Chem. 1993; 47:277-283.
- 282 19. Handa SS, Khanuja SPS, Longo G, Rakesh DD. Extraction technologies for medicinal
283 and aromatic plants. International Centre for Science and High Technology ICS-
284 UNIDO. Trieste, Italy. 2008.
- 285 20. Odimegwu D, Ibezim E, Esimone CO, Nworu C, Okoye F. Wound healing and
286 antibacterial activities of the extract *Dissotis theifolia* (melastomataceae) stem
287 formulated in a simple ointment base. J of Med Plant Res. 2008; 2: 11-16.