

1 **Raman Spectroscopic comparative study of Oxytocin and Freeze-dried Extract**
2 **of *Uvariadendron anisatum* Verdeck (*Annonaceae*) and their influence on diet**
3 **induced obesity in Sprague Dawley rats**

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5 Zephania Birech^{1*}, Peter W. Mwangi², Prabjot K. Sehmi², Nelly M. Nyaga²

6 ¹ Department of Physics, University of Nairobi, 30197, Nairobi, Kenya

7 ² Department of medical Physiology, University of Nairobi, 30197, Nairobi, Kenya

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9
10 *Corresponding author: birech@uonbi.ac.ke

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12 **Abstract**

13 Obesity is a condition affecting many people in the world. Obese people have increased risks of
14 developing chronic metabolic diseases such as type II diabetes, hypertension, cancer among others.
15 Early and rapid diagnosis of the condition together with effective treatment is therefore necessary.
16 This work investigated, first, Raman spectroscopic similarities between oxytocin and a freeze-
17 dried extract of a local herbal plant exhibiting oxytocin-like properties called *Uvariadendron*
18 *anisatum Verdeck (Annonaceae)* (UAV). Secondly, whether Raman spectroscopy could be used
19 for comparative studies of the influence of oxytocin and UAV on obese Sprague Dawley (SD) rat
20 models. We also wanted to find a Raman biomarker band for obesity or metabolic syndrome. Both
21 oxytocin and extract samples together with blood extracted from the rats were excited using a 785
22 nm laser after being placed or applied onto a conductive silver paste smeared glass slides and
23 Raman signals collected, recorded and analyzed.

24 The Raman spectroscopic spectral profiles of oxytocin and UAV freeze dried extracts were found
25 to be identical showing they were composed of similar Raman active molecules. The prominent
26 peaks were those assigned to disulfide S-S stretching mode at 508 cm^{-1} and to tyrosine at 645 cm^{-1} ,
27 846 cm^{-1} and 1617 cm^{-1} . Raman spectra of blood from rats treated with oxytocin and UAV had
28 indistinguishable profiles thus supporting idea that they were composed of similar active
29 molecules. The spectral profiles were also dissimilar to those from obese and non-obese (normal
30 controls) animals. A prominent peak in spectra of treated rats centered at 401 cm^{-1} could be used
31 as oxytocin biomarker band. Comparison of average intensity trend of fructose bands at around
32 638 cm^{-1} and 812 cm^{-1} between prepared fructose solution and blood of treated rats, revealed
33 elevated levels of fructose in blood of rats orally administered oxytocin and UAV extracts. The
34 implication was that fructose metabolism in rats administered oxytocin and UAV extracts was
35 upregulated. Principal component analysis (PCA) showed the power of Raman spectroscopy in
36 distinguishing between obese and non-obese SD rats based on spectral profile patterns. It also
37 further revealed that oxytocin and UAV extracts had similar influence on SD rats as their blood's
38 Raman spectral patterns were indistinguishable.

39 The study revealed that Raman spectroscopy can be a powerful tool for quick obesity (metabolic
40 syndrome) screening with intensity of Raman bands associated with fructose as biomarkers. The
41 same bands can also be used in comparative efficacy studies of anti-obesity drugs. Further studies
42 are needed to validate these Raman spectroscopic results since, to the best of our knowledge, this

43 was the first such investigation regarding comparison of UAV and conventional oxytocin together
44 with their influence on obese SD rats. Also studies on whether the same results can be seen in
45 human subjects.

46

47 **Keywords:** Oxytocin; Metabolic syndrome; Obesity; *Uvariodendron anisatum* Verdeck
48 (*Annonaceae*); Fructose

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50 **1. Introduction**

51 Obesity, a metabolic condition characterized by abnormal increase in body weight and fat
52 accumulation [1–3], is now a problem globally. According to World Health Organization (WHO),
53 it was estimated that by year 2016 about 650 million people worldwide were obese [2]. The
54 condition is caused by overconsumption of energy dense foods followed by less physical activity.
55 There is a close relationship between being overweight and being obese. The two i.e. obesity and
56 overweight are distinguished by a value known as body mass index (BMI) which basically is a
57 ratio of weight (in kilograms) to the square of height (meters squared). An overweight and an obese
58 human has a BMI value equal or greater than 25 and 30 respectively [2]. An obese person, therefore,
59 is overweight. In rodent models, there is no universally agreed method of determining obese from
60 non-obese rats but often those with fasting blood glucose (FBG) levels above 7 mmol/L [4] and
61 those with increased volumes of subcutaneous and visceral adipose tissues [3] are regarded as
62 obese. An obese individual has risks of developing chronic metabolic diseases such as type II
63 diabetes, hypertension, coronary heart disease, cancer among others[1,3,5]. Management of this
64 metabolic condition involves use of anti-obesity drugs, increase in physical exercise, reduction of
65 high energy diet. These methods are un-popular due to side-effects and high failure rates. New
66 interventions involving natural products with few side effects along with quick diagnostic
67 techniques for monitoring their efficacy and at the same time detecting potential development of
68 the condition are necessary.

69 One of the non-conventional potential alternative obesity treatments gaining a lot of attention lately
70 involves use of oxytocin[6–8]. Oxytocin (OT) is a hormone associated with labor, lactation [9–11]
71 and regulation of social behavior in mammals [10,12]. It has chemical formula $C_{43}H_{66}N_{12}S_2$ and
72 is locally produced in the brain and released to the circulatory system[12,13]. The compound
73 consists of nine amino acids in the sequence: cysteine - tyrosine - isoleucine - glutamine -
74 asparagine - cysteine - proline - leucine - glycine (CYIQNCPLG)[14]. The role of OT in weight
75 reduction in obese rhesus monkeys[6] and in rats[7,15] has been reported. In mice[16] and male
76 humans[16], OT caused a decrease in calorie intake. It has also been found that the hormone
77 suppresses eating behaviour resulting in reduction of blood glucose levels, increase in insulin
78 levels [10,17,18], reduction of glucose intolerance and insulin resistance[7] and a shift in diet
79 preference from carbohydrates to fats[16]. The hormone is also reported to make cells resistant to
80 diabetic conditions [13] and improve their insulin sensitivity [18]. In a study on African American

81 males, the levels of oxytocin in blood of type II diabetic subjects were found to be lower than in
82 healthy ones[19]. In the same study, subjects with higher levels of OT had lower body weights.
83 Administration of OT is through intranasal [12,16], intraperitoneal [20] and subcutaneous[16]
84 routes. Oral administration is rare due to its impaired and unpredictable absorption rates in the
85 gastric system [21] though a review of it is being suggested elsewhere[22].

86 All these findings indicate a special role OT plays in the treatment and prevention of obesity and
87 diabetes. Extended studies are, therefore, needed to investigate the influence of OT on metabolic
88 diseases and other potential uses in non-conventional treatments.

89 In many parts of remote rural Kenya where hospital facilities are distant, traditional herbalists and
90 birth attendants use herbal extracts for labor induction just as oxytocin. One of the herbs commonly
91 used, and which is the subject of our study is *Uvariadendron anisatum* Verdeck (*Annonaceae*)
92 (UAV)[23,24]. This work sought to investigate first, similarities of UAV freeze dried extracts and
93 oxytocin using Raman spectroscopy and secondly their influence on diet induced obesity in
94 Sprague Dawley (SD) rats. This study, to the best of our knowledge, was the first of its kind. It
95 was found that little Raman spectral differences exist between oxytocin and UAV extracts and no
96 distinguishable differences were observed on their influence on obese SD rats. Their
97 administration resulted in elevated levels of fructose in blood as revealed by intensity analysis of
98 assigned Raman bands.

99 **Experimental**

100 *Plant collection and extract preparation*

101 Fresh whole plants of *Uvariadendron anisatum* Verdeck (*Annonaceae*) were collected from their
102 natural habitat in Meru county, Kenya. The plant was confirmed at the University of Nairobi
103 herbarium and a voucher specimen deposited. The plant materials were air dried for a week before
104 being milled and grounded into powder. The powder (1 kg) was macerated in distilled water in a
105 weight to volume ratio of 1:8 for twenty (20) minutes and 8 litres of solution made. The resulting
106 suspension was then filtered using cotton wool and followed by Whatman's filter paper. The
107 resulting filtrate was frozen and lyophilization done to obtain freeze-dried extract. The freeze-dried
108 extract was weighed, placed in amber colored sample bottles and stored in a deep freezer.

109 *Animal experiments*

110 Twenty freshly weaned Sprague Dawley (SD) rats weighing around 95 g were used in the
111 experiment. They were housed, 5 members each, in metallic cages (dimensions 109 cm by 69 cm

112 by 77.5 cm) with floor covered with wood shaving. The shavings were replaced thrice every week.
113 Lighting of the cages was maintained at a 12-hour day and night cycle. For the first 8 weeks, all
114 the animals were fed on a high fat (15%) and high fructose (20%) diet *ad libitum*. Weight and
115 Fasting (5 hour fast) blood glucose levels including oral glucose tolerance tests were measured on
116 both day 0 and day 56 (last day of 8th week). On day 1, the animals were confirmed to be non-
117 diabetic as the FBG levels were on average 4.38 +/- 0.33 mmol/l which was less than 7.5 mmol/L,
118 a limit suggested by Wang *et al*[4]. The blood drawn was hence labelled as non-obese (Nob) and
119 stored. The weights and blood glucose level values (averaged 325 g and 6 mmol/L respectively)
120 obtained on the 56th day were used to designate the rats as obese. The rats were then regarded as
121 obese (with metabolic syndrome). These animals were thereafter randomly grouped, with 5
122 members each, into: Obese (Ob; fed on high fat 15%, high fructose 20% diet *ad libitum* as before),
123 Oxytocin treated (Oxy; same feeding as obese and administered oxytocin 1 mg/kg),
124 *Uvariadendron anisatum* Verdeck (*Annonaceae*) (UAV) extract treated at low dose (LDOx; same
125 feeding as obese and administered a dose of 100 mg/kg) and high dose (HDOx; same feeding as
126 obese and administered a dose of 200 mg/kg). The oxytocin and UAV treatment was carried out
127 for 7 days. Blood glucose testing was done using a commercial glucometer (StatStrip Xpress Nova
128 Biomedical, Waltham MA, USA) and weight measurement using an electronic beam balance. The
129 solvent used in dissolving both the oxytocin powder (Sigma-Aldrich, USA) and the freeze-dried
130 UAV extracts was normal saline (0.9% NaCl in water). All the prepared solutions of oxytocin and
131 the extracts were administered daily by oral gavage. The blood samples (~50 µL) were drawn from
132 each rat via lateral vein sampling after local anesthesia of the tail by topical application of lidocaine.
133 All the rats were then euthanized following an overnight fast using 20% Phentobarbital (1ml/kg
134 of body weight) injected intraperitoneally at the end of the experiment. Confirmation of death was
135 via loss of the pupillary light reflex. The drawn blood from each rat was stored in sodium citrate
136 vacutainers to prevent clotting and refrigerated at 4°C.

137

138 *Raman spectroscopy*

139 Raman spectroscopy was carried out using confocal Raman system (STR, Seki Technotron Corp)
140 equipped with a 785 nm laser and a spectrometer (Princeton Instruments). The conductive silver
141 paste smeared microscope glass slides used as Raman sample substrates were prepared as
142 described in [25]. Spectral calibration of the Raman spectroscopic device was also done as

143 described in reference [25]. The experimental parameters were: grating, 600 groves/mm; Centre
144 wavelength, 850.97 nm (980 cm^{-1}); exposure time, 10 sec; spectral accumulation, 5 sec;
145 microscope objective, X10 Max Plan. A small amount of blood ($\sim 10\ \mu\text{L}$) was pipetted onto the
146 silver smeared glass slide. Ten spectra per rat's blood sample were recorded making a total of 250
147 (50 data sets for non-diabetic samples included) spectral data sets with each group having 50 data
148 sets. Pre-processing of the data was done as described by Birech *et al*[25], analysis and plotting of
149 the spectral data were achieved using MATLAB 2017a and ORIGIN (Originpro 9.1) software.

150 2.4 Ethical approval

151 Ethical approval for the study was granted by the Biosafety, Animal Care and Use Committee,
152 Faculty of Veterinary Physiology, University of Nairobi.

153

154

155 2. Results and Discussion

156 2.1 Raman spectra of oxytocin and freeze-dried extracts of *Uvariadendron anisatum* Verdeck 157 (*Annonaceae*) (UAV)

158 The Raman spectra from UAV freeze dried extracts and those from commercially available
159 oxytocin displayed identical profiles and so indicating similar molecular composition (see Fig. 1).

160 The discrepancy in the spectral profiles was only observed from the different forms of the sample
161 (i.e. solid or solution). The spectra of oxytocin solution and UAV extract's dry powder (oxytocin
162 powder and UAV extract's solution) displayed identical spectral profiles as seen in Figure 1a (1b).

163 The exact reason why the extracts solids and oxytocin solution (also extract's solution and
164 oxytocin powder) had similar Raman spectral profiles is still unclear. It was thought that the
165 interaction between the silver smear and the samples are responsible for the observed variations.

166 The interactions must have influenced conformations of the various bonds in the oxytocin hormone
167 (which is composed of nine amino acids i.e. it is a nanopeptide) [14]. The most affected was the
168 disulfide S-S stretching mode at 508 cm^{-1} in the oxytocin powder resulting in red-shifting to 401
169 cm^{-1} in the extract's solution [14,26]. These same signals were observed to be broad in Figure 1a.

170 The conformational angle must have been less than 60° about the C-S bond as was argued earlier
171 by Maxfield and Scheraga [14]. The other prominent bands observed were those centered at
172 wavenumbers 645 cm^{-1} , 846 cm^{-1} and 1617 cm^{-1} ascribed to tyrosine with the commonly known

173 830/850 cm^{-1} doublet seen in oxytocin powder and solution [26,27]; 1240 cm^{-1} assigned to amide
174 III with anti-parallel β -sheet structure [26]; 1450 cm^{-1} assigned to C-H deformation in isoleucine
175 and 1658 cm^{-1} attributed to amide I vibrations with anti-parallel β -sheet conformation [26].

176 Figure 1. Figure displaying Raman spectra obtained from (a) UAV extract's dry powder and oxytocin
177 solution and (b) UAV extract's solution and oxytocin powder. All the samples were placed on
178 conductive silver paste smeared glass slides.

179 2.2 *Raman spectra of blood from SD rats*

180 The Raman spectra of blood obtained from SD rats that were obese (Ob), non-obese (NOB), obese
181 and administered oxytocin (Oxy), obese and administered UAV's extract at low dose (LDOx) and
182 high dose (HDOx) are displayed in Figure 2a. The intense peak at 401 cm^{-1} also seen in extract's
183 solution (see Fig. 1b) was present in all blood from rats administered oxytocin and UAV extracts
184 but less significant in obese and non-obese rats. This band may be used as oxytocin biomarker
185 band in blood and reflects elevated levels of the hormone in the treated animals. In other murine
186 studies, subjects administered oxytocin exhibited increased levels of the hormone (i.e. oxytocin)
187 in serum [10] and in plasma [7] thus supporting our observation through the assigned Raman peak.
188 Elsewhere, it was reported that in human subjects that were obese and with type II diabetes mellitus,
189 levels of oxytocin were significantly lower compared to healthy subjects [8,19]. The band centered
190 at 478 cm^{-1} was associated with both fructose and glucose's skeletal vibrations with tentative
191 assignments; C-C-C, C-C-O, C-O deformations and C-C torsional vibrations [28]. The bands
192 centered at 638 and 812 cm^{-1} were attributed to fructose and tentatively assigned to ring
193 deformation and C-C stretching vibrations respectively [28]. Interestingly, these two latter bands
194 (fructose bands) exhibited a decrease in intensity upon administration of both oxytocin and UAV
195 extracts to the diabetic rats as seen in Figures 2b and 2c. In order to interpret this trend, solutions
196 of fructose in normal saline were prepared with concentrations ranging from 0.005 – 0.015
197 mMol/L and Raman spectra obtained after pipetting onto the conductive silver coated glass slides.
198 The trend of the average intensity of peak centered around 812 cm^{-1} as a function of fructose
199 concentration (see Figure 2d) was identical to that from blood of SD rats (Figure 2b and 2c). The
200 implication of this was that fructose levels in blood of obese rats are lower than in non-obese and
201 treated rats (both oxytocin and UAV extract treated). At the same time, oral administration of
202 oxytocin and UAV extracts causes elevated levels of circulating fructose in SD rats.

203 Figure 2: Figure showing (a) Average Raman spectra from blood of obese(Ob), non-obese (NOB),

204 oxytocin treated obeserats (Oxy) and UAV extracts treated obese rats at low dose and high dose (LDOx
205 and HDOx) SD rats (b) and (c) Average Raman intensity of peak centered at 812 cm^{-1} and 638 cm^{-1}
206 respectively and (d) Average Raman intensity of peak centered around 812 cm^{-1} from fructose solution
207 in normal saline.

208 Here, Raman spectroscopic study indicates that fructose metabolism in the liver [29–31] is
209 upregulated by oral administration of oxytocin and UA extracts hence the increased concentration
210 in blood. It should also be noted that during treatment, the animals were still on a high fat and high
211 fructose diet. The high levels of fructose in blood are usually filtered out through the kidneys and
212 it is expected that their levels in urine are high as reported elsewhere in diabetic humans[32]. In
213 other studies, intraperitoneally injected oxytocin on mice resulted in reduced fructose
214 concentration in seminal vesicles and coagulating glands [20]. It was not clear presently whether
215 the method of administration of the extract and oxytocin brings about fructose level variations in
216 different body organs. Oral administration of oxytocin is unpopular due to impaired or
217 unpredictable absorption rates in the gastrointestinal tract [21,33]. The work here, therefore,
218 suggests that intensity of Raman spectral bands at 638 and 812 cm^{-1} assigned to fructose could be
219 used in quick indication of fructose level variation in oxytocin treated subjects and in obesity (or
220 metabolic syndrome) screening. The band can be used also in comparing anti-obesity influence of
221 conventional oxytocin and similarly used traditional plant extracts. The other bands centered at
222 1033 , 1130 , 1318 and 1443 cm^{-1} are associated with the branched chain amino acids (BCAAs).
223 The peaks centered at 1033 and 1130 cm^{-1} are ascribed to C-N stretch, NH_3 rocking, HCCH
224 torsional vibrations in leucine [34]; CO stretch, OH bending vibrations in both valine and
225 isoleucine [34].

226 2.3 *Principal component analysis (PCA)*

227 When Raman spectroscopy is to be used to make quick examination of influence of oxytocin and
228 UA extracts on obesity, a method to distinguish between spectral profiles from the different blood
229 samples is needed. In this work, principal component analysis (PCA) was used. The method
230 utilizes spectral patterns in segregating between spectral data. The spectral pattern variations are
231 expressed in terms of percentage variance and ranking done[35,36]. The results are represented on
232 a set of orthogonal axes referred as principal components (PCs). The PC with the highest variance
233 is called PC1, followed by PC2 and so on [35]. Each of the spectral data set is displayed as a point
234 (score) on a PC plane. For our work it was found that Raman spectral data from blood of obese,

235 non-obese were clearly differentiated from each other and from the rats administered oxytocin and
236 UAV extracts (Oxytocin, low dose and high dose) as displayed in Figure 3. The low and high doses
237 of the UAV extract did not show distinguishable differences in the score plot.

238 Figure 3: Figure displaying PCA score plot from Raman spectral data of SD rat's blood. PC1 and PC2
239 had explained variance of 74.7% and 9.5% respectively. The spectra from obese rats were clearly
240 distinguished from non-obese and the treated rats.

241 The results of the study indicate that Raman spectroscopy can be used as a label free obesity
242 detector or screener with bands associated with fructose as biomarkers. At the same time, the
243 results show that Raman profiles from blood of oxytocin treated rats and those treated with UAV
244 extracts contained similar Raman active molecules. The two compounds (i.e. oxytocin and UAV
245 extracts) influenced obesity in the rats since the spectral profiles were modified. This was also
246 supported by the fact that the average weights and FBG values of the treated animals decreased
247 (325 g to 260 g and 6.3 +/- 0.3 mmol/L to 4.7 +/- 0.4 mmol/L respectively) in the first 7 days after
248 commencing treatment. The low and high doses of the UAV extract did not exhibit discernible
249 differences on the rats as the blood had identical profiles. Herbalists and traditional birth attendants
250 in parts of rural Kenya use UAV to induce labor [23,24]. The Raman study results reveals that the
251 herb has identical effects in obese SD rats as the conventional oxytocin. This implies that the herb
252 is composed of similar Raman active molecules. Further studies need to be done to validate the
253 Raman spectroscopic results reported here as this, to the best of our knowledge, is the first such
254 investigation as regards comparison of UAV and conventional oxytocin and on their influence on
255 obesity in rats.

256

257

258 **3. Conclusion**

259 The study revealed that Raman spectroscopy can be a powerful tool for comparative study of anti-
260 obesity drugs as spectral profiles from obese, non-obese and treated rats were distinguishable. The
261 peaks associated with fructose could be used as biomarker bands for the distinction. The method
262 further showed that oxytocin and UAV are composed of identical Raman active molecules and
263 possesses similar anti-obesity effects. They both also cause elevated levels of fructose in blood of
264 rats.

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Raman Intensity /Arbtr. Units

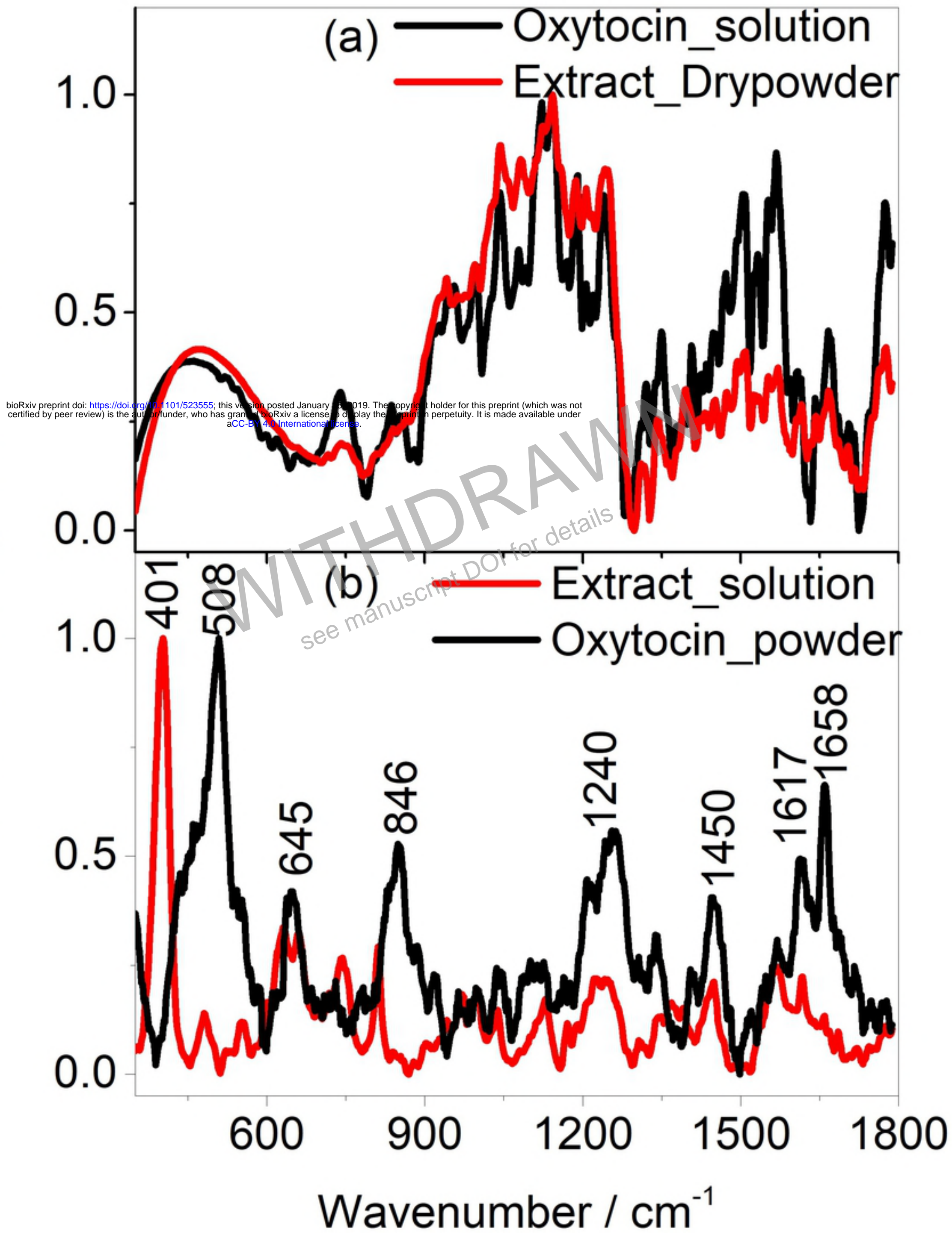


Figure 1

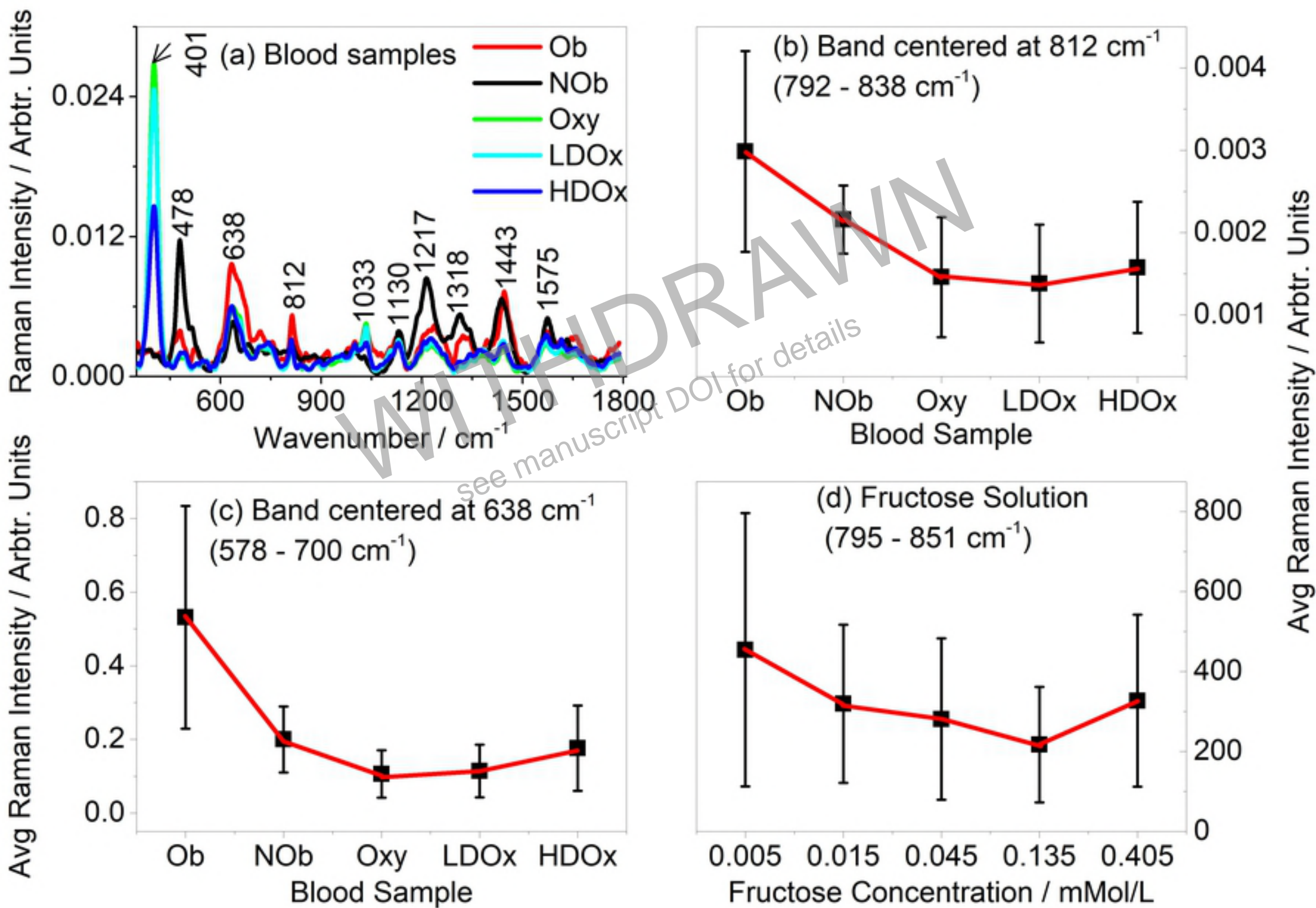


Figure 2

PC1 (74.7%)

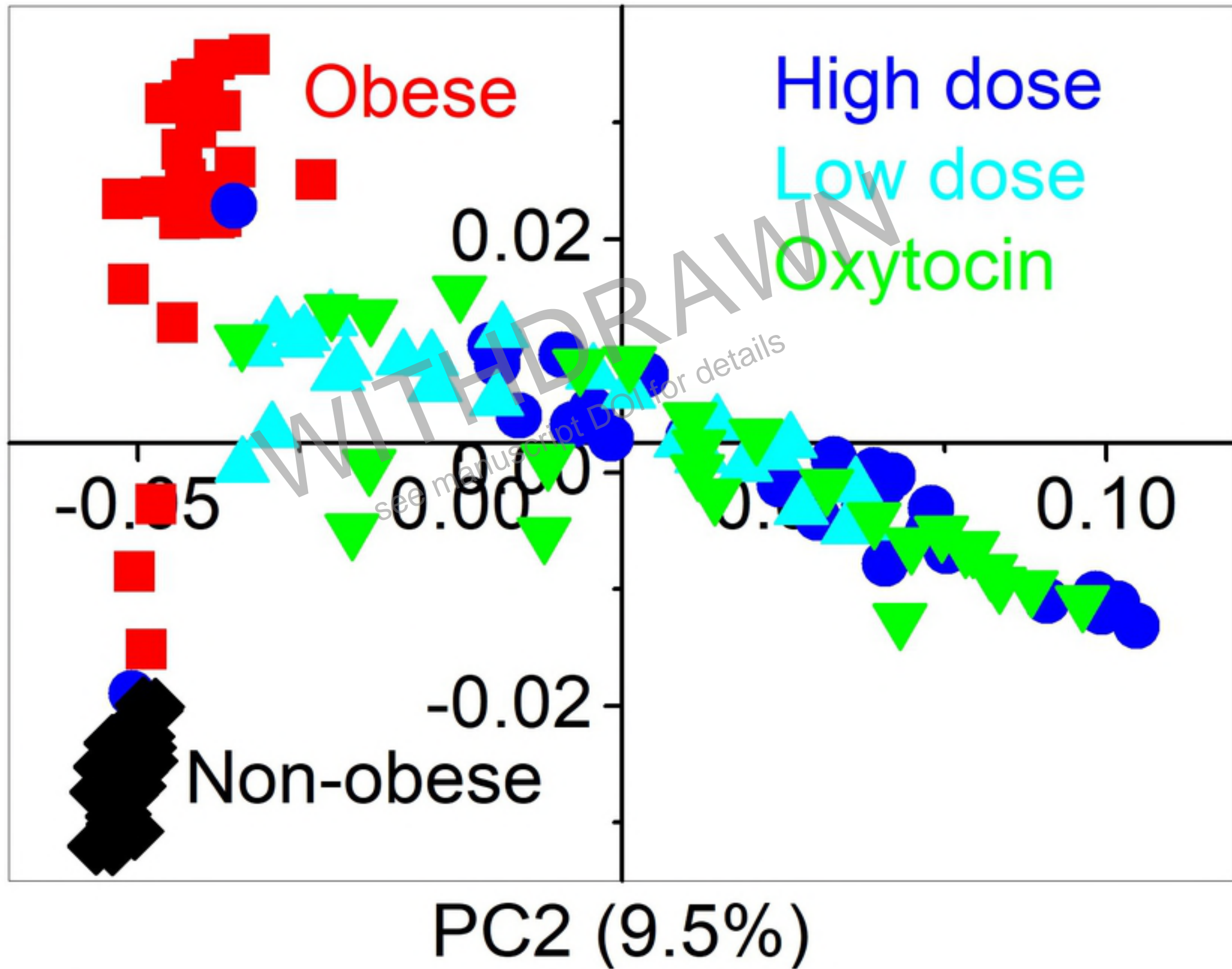


Figure 3