



Received on 01 July, 2016; received in revised form, 30 August, 2016; accepted, 26 October, 2016; published 01 January, 2017

ANTIMICROBIAL ACTIVITIES OF PHARMACOLOGICAL AND ANTIOXIDANT COMPOUNDS OF SOME EDIBLE MUSHROOMS AGAINST *BRUCELLA* SPP.

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Keywords:

Edible Mushrooms,
Antimicrobial activity,
Brucella spp., Antioxidant capacity

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ABSTRACT: Although there are numerous studies on antimicrobial activities of mushrooms against many common pathogenic microorganisms, there is hardly any study against *Brucella* spp., responsible for brucellosis in animals and human beings throughout the globe. The present study was aimed to evaluate the *in vitro* antimicrobial activities of ethanol extracts of five edible mushrooms namely, *Pleurotus ostreatus*, *Pleurotus florida*, *Pleurotus eous*, *Pleurotus sajor-caju* and *Calocybe indica* against four reference strains of *Brucella* spp.: *B. abortus* S19, *B. abortus* S99, *B. melitensis* M16 and *B. suis* 1330. Methodologies adopted were disc diffusion assay, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts. Evaluation of the antioxidant capacity of the extracts of the mushrooms by DPPH free radical scavenging activity was also aimed in this study. The total phenol content of the mushrooms showed significant positive Pearson correlation with the antioxidant capacity of the extracts. The results obtained revealed the antimicrobial potency of all the screened mushrooms against *Brucella* spp. *P. sajor-caju* had highest phenolic content, which may be responsible for its highest antioxidant capacity resulting outstanding antimicrobial activities. This study signifies the possible use of these edible mushrooms as source of effective antimicrobial agents against brucellosis.

INTRODUCTION: Although medical fraternities could consider new generation antibiotics, as life saving end products, against complicated bacterial infections; but most pharmaceutical industries has discontinued research activities for development of newer antibiotics, due to fewer profit margins.

This is due to unexpectedly rapid development of microbial resistance, against newly developed antibiotics in recent past, imposing a threat not only to all the clinicians around the globe, but also to mankind as a whole. In 2015 and 2016 many microorganisms are being reported as having MCR-1 gene, which causes resistance to the most effective antibiotic colistin, which is commonly used in terminal patients with infections ¹. This global extend of drug resistance also accomplished the intracellular pathogens like *Brucella*, with limited number of known antimicrobials effective against them, indicating an added threat in near

<p>QUICK RESPONSE CODE</p> 	<p>DOI: 10.13040/IJPSR.0975-8232.8(1).323-29</p> <hr/> <p>Article can be accessed online on: www.ijpsr.com</p>
<p>DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.8(1).323-29</p>	

future, as few workers are engaged in studies with these microorganisms, due to high risk of laboratory acquired infections, and requirement of higher biological safety level precautions^{2,3}. It was evident from the literature survey that strains of *B. abortus* were found to be resistant to eight antimicrobial agents, which are commonly used to treat patients with human brucellosis.

A study showed strains of *B. abortus* to be highly resistant to azithromycin (100%), sulphamethoxazole-trimethoprim (98.9 %), moxifloxacin (80.7 %) and ciprofloxacin (69.3 %). The same study showed comparatively low resistance against rifampicin (6.8 %), Streptomycin (5.7 %), tetracycline (1.1 %) and doxycycline (1.1%)⁴. Again, classical drug combination treatments against brucellosis, along with their side effects, such as high gastric discomfort, high relapse rates and therapeutic failures, entail necessity of newer antimicrobials to mitigate these highly virulent agents⁵. Therefore, as an alternative and complementary approach, many medicinal plants and other herbal resources are getting explored everywhere, to discover a novel antibiotic with a new mode of action. In this regard, mushrooms can definitely be considered as a potential source of new antimicrobial compounds, and it has been noticed that there is a recent rise in interest among scientists regarding mushrooms, not only as a healthy food, but also for its bioactive compounds, which are getting used as complementary medicine or dietary supplements for anticancer, antiviral, immune stimulating, hypocholesterolemic and hepatoprotective activities⁶. For example, mushrooms such as *Ganoderma lucidum*, *Lentinula edodes*, *Lepista nuda* and many others, are classified as medicinal mushrooms, and recent studies on various other mushrooms have also reported that different types of their extracts showed broad spectrum antibacterial activities against common pathogenic bacteria^{7,8}.

The mushrooms are also well known to gear up the immune system, as they consist of immune modulatory polysaccharides-protein complexes, and thus may have an added benefit to prevent relapses⁹. Brucellosis is a zoonotic disease, and it mainly occurs accidentally in humans, who live in the close proximity of the infected animals, or after

consuming infected animal products, such as unpasteurized milk and milk products. In case of human brucellosis, infections due to *B. melitensis* are higher, followed by *B. abortus* and *B. suis*. Rare cases have been observed with infection due to *B. canis*^{10, 11}. The present work was done with a motive to screen for the first time, the *in vitro* sensitivity of *Brucella* spp., to edible mushrooms extracts of *P. ostreatus*, *P. florida*, *P. sajor-caju*, *P. eous* (oyster mushrooms) and *C. indica* (milky mushroom).

MATERIALS AND METHODS:

Collection and processing of mushroom

Samples: Samples of mushrooms – Fresh samples of oyster mushrooms *Pleurotus ostreatus*, *Pleurotus florida*, *Pleurotus eous*, *Pleurotus sajor-caju* and milky mushroom *Calocybe indica* were collected from a reputed mushroom cultivation farm, maintained by Ramakrishna Mission, Narendrapur, Kolkata, India. The fresh samples were dried in shade for three days, powdered, and stored in clean dry air tight containers.

Collection and maintenance of microorganisms:

Four reference strains of bacteria *Brucella abortus* S19, *Brucella abortus* S99, *Brucella melitensis* M16 and *Brucella suis* 1330 were obtained from National *Brucella* Repository Laboratory (IVRI, Izatnagar, Uttar Pradesh, India). The fastidious microorganisms were maintained in stock by subculturing on *Brucella* selective agar base.

Extract preparation: Fresh mushroom samples were shade dried, and then powdered, which was later extracted with 60 per cent (v/v) ethanol for about 72 hours in dark, at room temperature. The extracts were then filtered with Whatman filter paper. Then all the extracts were sterilized with 0.22 micron syringe membrane filter, after which it was lyophilized, and the crude bioactive content was measured and was freeze dried¹².

Antibacterial screening assays: The antimicrobial activity was screened through agar disc diffusion method, and minimum inhibitory concentration assay (MIC), by serial dilution method, using 96 well plate, and plate reader (Erba Lisa Scan II Transasia Mannheim, Germany). The minimum bactericidal concentration (MBC) was performed

following determination of the MIC concentration. After completion of MIC, an aliquot from each well of the serial dilutions was inoculated on enriched *Brucella* selective agar base. The plates were examined, and CFU/mL was counted after incubation in 5 per cent carbon dioxide environment. The plate showing 99.9 per cent inhibition, in comparison to the initial count of the original inoculums, was considered to be as MBC. For all the assays 0.5 McFarland opacity culture was used^{13, 14, 15}. All the assays were carried out in triplicate.

Determination of bioactive components by phytochemical screening assays: The bioactive components of the extract were previously screened by the phytochemical screening assays using standard protocols¹⁶. Tests were conducted for flavonoid, terpenoid, steroid, tannins, saponins. The total phenol content and the total flavonoid content was measured using standard protocol of Folin Ciocalteu method and aluminium chloride assay.

Determination of DPPH free radical scavenging activity: Antioxidant activity of a sample can be measured principally by electron transfer reaction mechanism. Here reduction of an oxidant, which shows change in color when reduced, is the principle mechanism of the reaction. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging

activity was measured following the modified method of Xu and Chang¹⁷. To 3.8mL of DPPH methanol solution (0.1mM) added 0.2mL of extract solution, and mixed by shaking it vigorously. It was allowed to stand in dark, for 30 minutes, and then the absorbance was measured at 520nm. The DPPH solution mixed with extraction solvent is kept as blank for the experiment. The per cent of DPPH discolouration is calculated by the formula:

$$\% \text{ of DPPH discolouration} = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] * 100.$$

Here A_{control} is the absorbance of the blank sample.

Statistical Analysis: The statistical analysis of the data was done using statistical software GRAPH Pad Prism Version 5. Results were expressed as mean of at least three separate determinants. The results were analyzed using one – way ANOVA. Significant differences were accepted at $P \leq 0.05$. Pearson correlation coefficient was used to determine correlation between total phenol or flavonoid content and antioxidant activity. Differences between means at P value < 0.005 level were considered significant.

RESULTS AND DISCUSSION: The results are given in the **Tables 1-2** and in **Fig. 1-12**.

TABLE 1: SHOWING THE ZONE OF INHIBITION OBTAINED FROM DISC DIFFUSION ASSAY

Microorganisms	<i>P. ostreatus</i>	<i>P. florida</i>	<i>P. eous</i>	<i>P. sajor-caju</i>	<i>C. indica</i>
<i>B. abortus</i> S19	++	+	+	+++	+
<i>B. abortus</i> S99	+	+++	++	+++	-
<i>B. melitensis</i> M16	+	-	+	+	-
<i>B. suis</i> 1330	-	+	+	+	+

Diameter of the zone of inhibition - : 10-15 mm (+); (++) 16-20 mm; (+++) 20-25mm; No inhibition

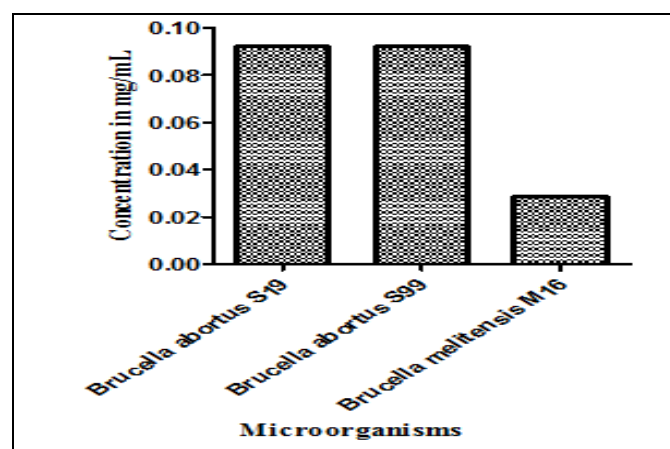


FIG. 1: MIC ANALYSIS OF ETHANOL EXTRACT OF *P. OSTREATUS* AGAINST *BRUCELLA* SPP.

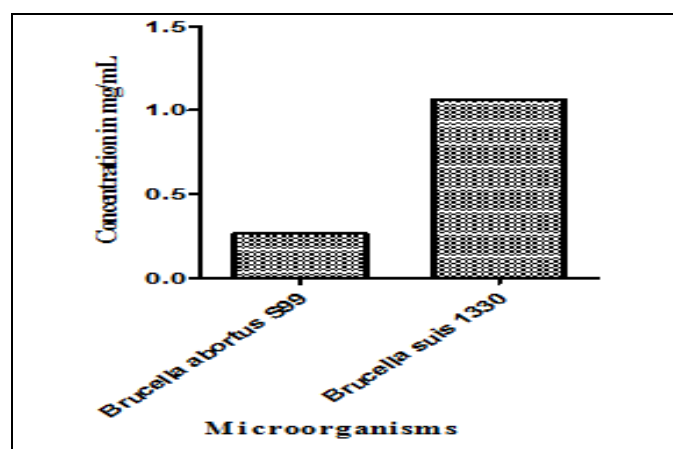


FIG. 2: MIC ANALYSIS OF ETHANOL EXTRACT OF *P. FLORIDA* AGAINST *BRUCELLA* SPP.

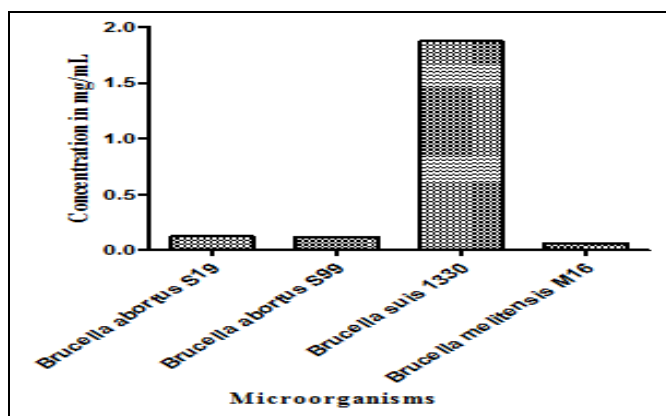


FIG.3: MIC ANALYSIS OF ETHANOL EXTRACT OF *P. EOUS* AGAINST *BRUCELLA* SPP.

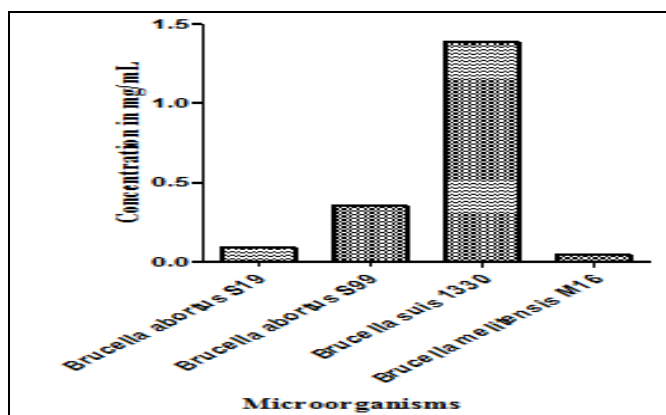


FIG. 4: MIC ANALYSIS OF ETHANOL EXTRACT OF *P. SAJOR-CAJU* AGAINST *BRUCELLA* SPP.

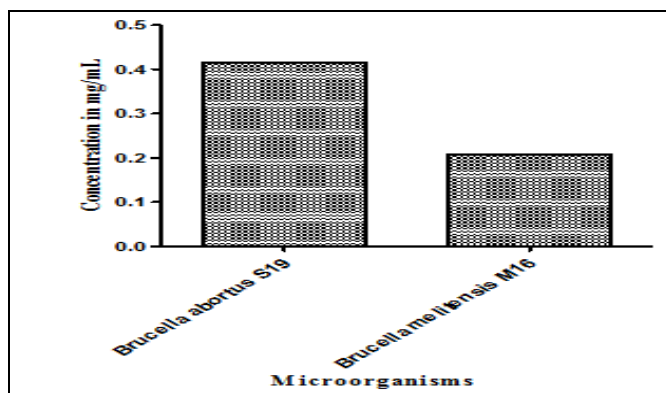


FIG.5: MIC ANALYSIS OF ETHANOL EXTRACT OF *C. INDICA* AGAINST *BRUCELLA* SPP.

FIG 1-5: THE BAR DIAGRAM REPRESENTS THE MEAN OF THE MINIMUM INHIBITORY CONCENTRATION (IN mg/ml) OF THE ETHANOL EXTRACTS OF MUSHROOMS AGAINST *BRUCELLA* SPP.

Antimicrobial assay: As evident from the data (shown in Table 1), the crude ethanolic extracts of all the five edible mushrooms have inhibitory effect, upon the growth of *Brucella* spp. In case of *P.ostreatus*, the antimicrobial activity was found to be satisfactory against *B. abortus* S19, *B.abortus* S99 and against *B.melitensis* M16 (Fig. 1 and Fig.

6); but it showed no activity against *B. suis* 1330. The other mushroom extracts *P. eous*, *P. sajorcaju* and *P. florida* showed potent inhibitory action upon all the strains of *Brucella* spp.(Fig.2-4 and Fig. 7-9), however, *P. florida* did not show any action against *B. melitensis* M16. The extract of *C. indica* showed inhibitory action upon *B.abortus* S19 and *B. suis* 1330(Fig. 10 and Fig 5). Previous studies revealed that the *in - vitro* inhibitory action of the widely used oyster mushroom *P. ostreatus*, and *P. sajor-caju* showed inhibitory action upon *Enterobacter aerogenes*, *Salmonella enterica* serotype Typhimurium, *Escherichia coli*, *Proteus vulgaris*. *P. sajor caju* also showed potent inhibition against *Klebsiella pneumoniae* ¹⁸.

Furthermore, the study on the antimicrobial action of extracts of *P. djamor* and *C. indica* has been found not only against *Pseudomonas aeruginosa*, *Proteus vulgaris* but also against *Candida albicans* ¹⁹. However, no previous study has explored the antimicrobial potency of the common edible mushrooms against *Brucella* spp.

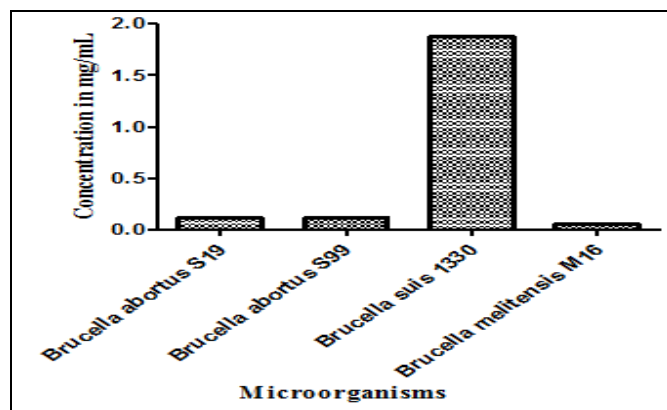


FIG.6: MBC ANALYSIS OF ETHANOL EXTRACT OF *P. OSTREATUS* AGAINST *BRUCELLA* SPP.

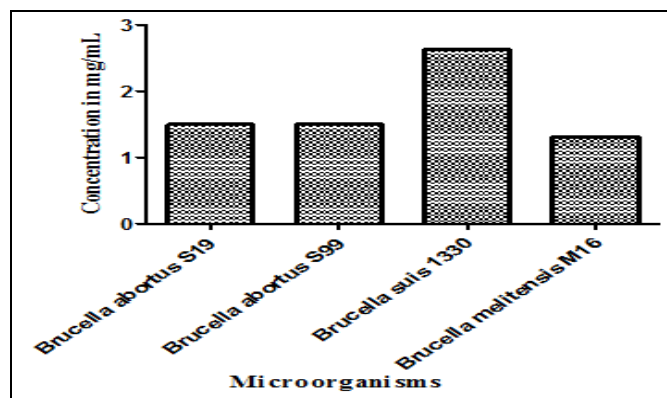


FIG.7: MBC ANALYSIS OF ETHANOL EXTRACT OF *P. FLORIDA* AGAINST *BRUCELLA* SPP.

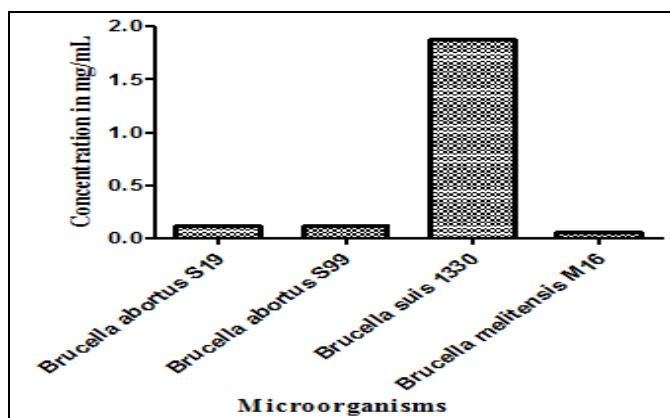


FIG.8: MBC ANALYSIS OF ETHANOL EXTRACT OF *P. EOUS* AGAINST *BRUCELLA* SPP.

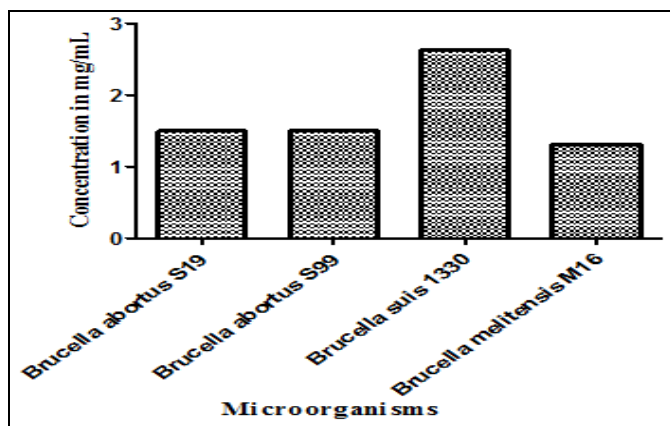


FIG.9: MBC ANALYSIS OF ETHANOL EXTRACT OF *P. SAJOR-CAJU S* AGAINST *BRUCELLA* SPP.

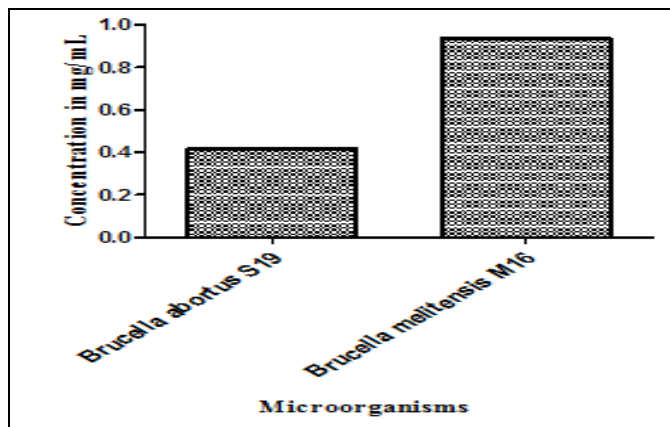


FIG.10: MBC ANALYSIS OF ETHANOL EXTRACT OF *C. INICA* AGAINST *BRUCELLA* SPP.

FIG 6-10: THE BAR DIAGRAM REPRESENTS THE MEAN OF THE MINIMUM BACTERICIDAL CONCENTRATION (IN mg/ml) OF THE ETHANOL EXTRACTS OF FIVE EDIBLE MUSHROOMS AGAINST *BRUCELLA* SPP.

Evaluation of bioactive components: As mentioned in our previous study the ethanol extracts of mushrooms contains several bioactive components such as phenol, terpenoid, steroid, flavonoid and saponins¹⁶. The presence of phenol and flavonoid content in the extracts may

contribute to the potent antimicrobial action of the mushrooms against *Brucella* spp.

Antioxidant assay: The extract of *P. sajor-caju* showed highest antioxidant activity and least activity was shown by the extract of *C. indica* based on the IC_{50} value (Fig. 11). In our study there is positive correlation (P value < 0.005) between the total phenol content and antioxidant capacity of the extracts of mushrooms (Fig.12) and this observation was previously reported by other authors in their study. However, there is no correlation between the total phenol content and total flavonoid content which is again in accordance with the result obtained from the same study. The phenol or flavonoid content varies species to species of mushroom²⁰.

As there is no single solvent which could extract all the major antioxidant compounds from mushrooms so extraction with 60 per cent ethanol -aqueous solvent was done to get a better yield as previous study had already reported significant increase in yield with mixture of organic solvent with water¹². The total phenol and flavonoid content extracted using 60 percent ethanol for about 72 hours showed increased yield than that of previously reported by others in their study using absolute ethanol for about 24 hours²¹.

This is a novel study regarding the antimicrobial action of mushrooms against *Brucella* spp. as no literature is available till date.

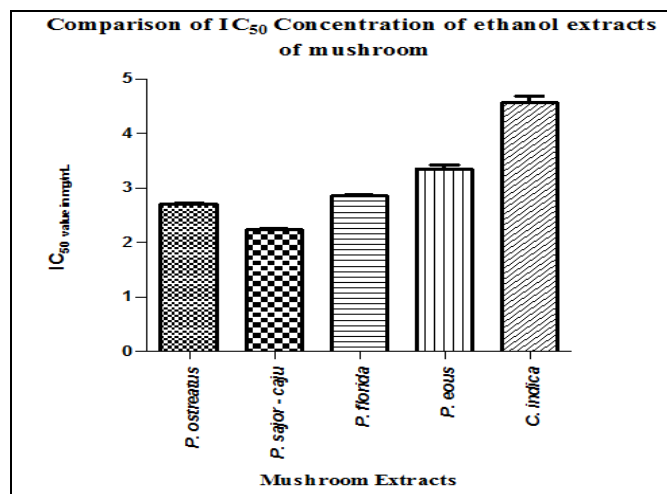


FIG. 11: THE BAR DIAGRAM REPRESENTS THE MEAN \pm SD OF ACTIVITY (IC_{50} in mg/mL) CONCENTRATION OF MUSHROOM EXTRACTS. THE RESULTS WERE FOUND SIGNIFICANT [P VALUE < 0.05 BY ONE - WAY ANOVA]

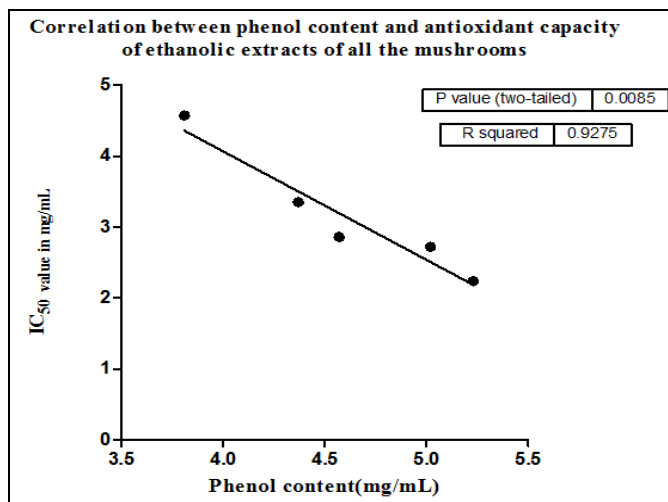


FIG. 12: DATA SHOWING POSITIVE PEARSON CORRELATION BETWEEN THE TOTAL PHENOL CONTENT AND IC₅₀ VALUE (mg/ml).

CONCLUSION: The study reveals a possible use of these mushrooms as newer antimicrobial agents in future, as the conventional treatments of brucellosis, which are currently available show a gloomy picture, marred with failures of treatment, and upsurge of recurrence of the disease. The bioactive components extracted during the extraction procedure, showed excellent antimicrobial activity particularly against strains of *Brucella abortus*, along with prominent antioxidant activity. As hardly any study is available regarding the antimicrobial activity of these edible mushrooms against microorganisms of the family Brucellaceae, and as the disease brucellosis is a chronic one, with high chances of relapses; so study on this field is of paramount importance, from microbiological and pharmaceutical point of view in near future.

CONFLICT OF INTEREST: There is no conflict of interest.

ACKNOWLEDGEMENT: The authors acknowledge the authorities of the mushroom cultivation farm of Ramakrishna Mission, Narendrapur, West Bengal, India, for providing the samples for research purposes. The authors would also like to acknowledge DST INSPIRE division, Government of India, for providing the financial assistance to Debasmitha Chatterjee throughout the work and to DBT Brucella sero-epidemiology network project, BE-8, Dept. of Science & Technology, Govt. of India for providing some technical facilities in this experiment.

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How to cite this article:

Chatterjee D, Halder D, Chakraborty U, Dutta D, Kuila P and Das S: Antimicrobial Activities of Pharmacological and Antioxidant Compounds of Some Edible Mushrooms against *Brucella* Spp., *Int J Pharm Sci Res* 2017; 8(1): 323-29. doi: 10.13040/IJPSR.0975-8232.8(1).323-29.

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